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13. ABSTRACT ***(Maximum 200 Words)***

In the present report we describe studies using several types of chimeric immune receptors. These receptors consist of an antibody fragment against the CEA tumor antigen joined to signaling portions of various T cell receptors (e.g., IgTCR, IgCD28, IgLFA1). The antibody fragment confers CEA specificity to the receptor, while the signaling domains transmit T cell activation and costimulation signals. These receptors allow the T cell to bypass immune tolerance to CEA and to activate effector functions in a tumor specific manner. We describe our progress in completing a phase I study using IgTCR alone. We also present in vitro proof-of-principle studies using combinations of different receptors. These studies demonstrate that specific signaling molecules activate specific T cell effector functions in a tumor specific manner. These studies identify the key immune regulatory elements necessary to reconstructing an effective anti-tumor immune response.

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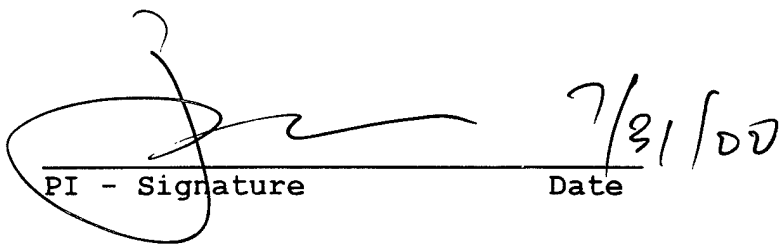

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4. Table of Contents

5. INTRODUCTION

Carcinoembryonic antigen (CEA) is a tumor-associated antigen which is expressed on ≈30-60% of metastatic breast tumors. The purpose of this research is to develop a new type of cancer therapy using autologous T cells modified with a chimeric immunoglobulin T cell receptor (IgTCR) directed against CEA+ tumors. The specific objectives are to:

1. Complete setup for therapy.
2. Apply IgTCR-modified cells in a phase I clinical study in patients with metastatic CEA+ breast tumors
    a. Determine the safety and tolerability of anti-CEA modified T cells.
    b. Describe the pharmacokinetics by the persistence of modified T cells in blood.
    c. Evaluate immunogenicity of the modified cells.
    d. Measure immunologic and other parameters which correlate with efficacy.
    e. Preliminary evaluation of efficacy.

In addition, to the above, we have also carried out basic research efforts to create second-generation reagents that will enhance the therapy for future phase II/III studies.

6. BODY:

In our last annual report we documented the completion of Specific Aim # 1, and the initiation of the clinical study (Aim #2). We also discussed progress made on developing second generation reagents to enhance the therapy. In the present report we outline the progress we have made since this prior report.

We have not treated any additional patients since our last report. This was due to a number of reasons. Following the treatment of the last patient, the study was placed on hold by our Institutional Review Board (IRB) in order to reevaluate patient safeguards. A Data Safety Monitoring Board (DSMB) was assembled and the protocol, informed consent document, eligibility requirements, and enrollment procedures were extensively reviewed. The DSMB made a number of recommendations and these were incorporated into our protocol and procedures. These recommendations included more stringent eligibility and exclusion criteria, more rigid dose modification criteria, more clearly defined criteria to either continue/discontinue therapy, and a more clearly defined period for toxicity monitoring. This period of IRB review lasted for approximately 2 months.

Following the IRB review, the study was reviewed by the Harvard Biosafety Committee (HBC). The HBC also audited the laboratory and the production facility used to manufacture patient T cells. Following their review, the HBC placed the study on hold until a number of changes were made to our facilities and procedures. The most significant change was the requirement that clinical production be carried out in a dedicated facility. When the clinical study was initiated, manufacturing was performed in a mixed use tissue culture facility (clinical and basic research). Prior approval for a mixed use facility had been obtained from the FDA. However, the HBC has elected to enforce standards that are significantly more restrictive. The HBC also requested a number of changes in recordkeeping, Standard Operating Procedures (SOPs), quality control mechanisms, and other more minor changes.

Much of the time period since the last report was spent obtaining and establishing a dedicated clincial-only production facility, and making other HBC-requested changes. This has involved a significant amount of work that, while not directly specified in the aims, was nonetheless unavoidable in our efforts to complete Specific Aim #2. We have recently completed these HBC-mandated changes and the modified protocol will be reviewed by the HBC at their next meeting. We anticipate that we will be given approval to proceed with the study following this review. All other regulatory agencies/boards have indicated their satisfaction and have released us from clinical hold. We have received a no cost extension and will complete the phase I study within the next 6-8 months.

We have completed several other portions of the clinical study that involve analysis of laboratory correlates. These includes analysis of vector immunogenicity (negative for all patients treated thus far) and retrospective analysis of drug safety parameters (replication competent retrovirus, mycoplasma, endotoxin). We have also developed and validated new assay systems that will streamline the required testing process and decrease the cost of treatment. We have provided the FDA with the additional manufacturing and validation

studies that would permit changing the standard regimen of tests and have been released form hold by the FDA. Again, although these activities were not directly specified in the Specific Aims, they nonetheless represent important accomplishments in achieving our overall goals of developing this technology through phase I studies.

Although we have been on clinical hold and unable to make progress on completing all patient treatmetns (Specific Aim #2), we have made significant progress on developing new reagents to improve the therapy. The development of these new reagents goes to the heart of the original hypotheses, which revolve around creating the constructs and procedures that will enable us to engineer effective anti-tumor immune responses. In accordance with this overall goal, we have conducted an extensive analysis of the immune functions activated by IgTCR molecules both alone and in combination with other T cell receptors involved in activating T cell responses (e.g., CD28, LFA1, CD2). These studies were conducted using normal peripheral blood mononuclear cells (PBMC). Most previous studies have indicated that IgTCR molecules alone are able to activate all effector functions (1-13). However, these previous studies relied on model systems rather than direct demonstrations in normal T cells.

Our studies (14-15) have clearly demonstrated that IgTCR molecules are not sufficient, by themselves, to fully activate anti-tumor immunity. We have shown that IgTCR molecules can effectively fulfill two important functions. First, they are able to impose anti-tumor specificity on normal T cells. Second, they confer potent, tumor specific cytolytic functions to the T cells. Tumor cell killing does not require costimulation or IL2 (14). However, in the absence of costimulation, IgTCR crosslinking induces activation induced cell death (AICD), much in the way TCR signals alone induce AICD in a native immune response (Fig. 1 A and C). IgTCR-induced AICD results in the death of all tumor-reactive T cells over a period of 7-10 days. Highly effective tumor cell killing occurs during this period, but the AICD renders the response unsustainable (Fig. 2). We have further shown that the combination of IgTCR and CD28 blocks this AICD and induces the rapid proliferation of tumor reactive T cell clones (Fig. 1B and C). This proliferation occurs in a tumor specific manner. Specifically, only the target tumor will induce proliferation, and only tumor reactive T cell subclones activate proliferative functions. Thus, combining IgTCR and CD28 signals induces immune responses that more closely mirrors events that take place during a native response. This mirroring includes an interesting phenomenon in which the gene modified T cells undergo a self-imposed, antigen-driven selection process that results in the preferential growth of subclones that have the highest affinity for the tumor target (Fig. 3). This is similar to the repertoire focusing and affinity selection that occurs during a normal immune response (16-19). The more complete immune response induced by combining IgTCR+CD28 (e.g., activation of tumor killing and T cell proliferation) increases anti-tumor activity by >200-fold (Fig. 2B and C). We are currently developing CD28 chimeras in order to utilize this advancement clinically. These studies have been published, or will be in the near future (reprint/manuscripts attached).

Although coupling CD28 and IgTCR signals leads to more complete anti-tumor immunity, the overall immune response is still lacking in comparison to the full potency of a native immune response. The hallmarks of an effective T cell immune response are the activation of cytolytic

functions, T cell proliferation, and cytokine release (particularly IL2 release). IgTCR and CD28 crosslinking activates cytolytic and proliferative functions (Figs. 1 and 2), but does not lead to effective IL2 release. Our studies have shown that IL2 release requires the engagement of a third receptor (Fig. 4). This third receptor can be either the LFA1 or CD2. Crosslinking either of these receptors leads to the release of enough endogenous IL2 to completely sustain the immune response (Fig. 5). Thus, the incorporation of Signal 1 (TCR), Signal 2 (CD28), and Signal 3 (LFA1, or CD2) induces an autonomous, and independently sustained immune response. We are currently developing Signal 3 chimeras in order to incorporate this third effector function (IL2 release) in clinical applications if necessary. A manuscript describing these Signal 3 studies is currently in preparation.

7. KEY RESEARCH ACCOMPLISHMENTS

- Determination of toxicity and tolerability in humans.
- Completion of portions of Specific Aim #2.
- Changes in facilities and procedures that will allow completion of remainder of Aim #2.
- Identification of the key elements of an effective anti-tumor immune response.
- Proof-of-principle for the relationships between different T cell activation signals and different immune effector functions.
- Development of second generation reagents to improve therapy.

8. REPORTABLE OUTCOMES

- Abstract and poster presentation at the Army's annual breast cancer conference.
- Invited speaker presentation at the annual meeting of the Society for Biological Therapy.
- U.S. Patent application
- Grant applications supported by the research:
  - NCI Howard Temin Award
  - U.S. Army prostate cancer research program
  - Susan Komen Breast Cancer Foundation
  - Cancer Research Institute
  - Aid for Cancer Research Foundation
  - Fireman Foundation (Finalist)
  - Mass. Dept. Public Health for breast cancer research
- Publications:

  Nolan, et. al. Bypassing immunization: optimized design of designer T cells against CEA-expressing tumors and lack of suppression by soluble CEA. Clin. Cancer Res. 3928:3928-3941.

  Beecham, et. al., Dynamics of tumor cell killing by T cells armed with an anti CEA chimeric immunoglobulin T cell receptor. J. Immunother. 23:332-343.

  Beecham, et. al., Coupling CD28 costimulation to IgTCR molecules: dynamics of T cell proliferation and death. J. Immunother. Submitted.

9. CONCLUSIONS

The results of these studies demonstrate that T cells modified with an anti-CEA IgTCR molecule are well tolerated in human cancer patients. Preliminary indications of anti-tumor efficacy in vivo suggest that modified T cells may induce weak anti-tumor responses. However, anti-tumor responses are not sustained for more than a few days, correlating with in vitro studies indicating that IgTCR modified T cells die from activation induced cell death (AICD) over a comparable time frame. An extensive analysis of various T cell activation receptors and the effector functions that they induce indicate that specific signaling receptors are responsible for turning on each of the major effector functions in a stepwise fashion. Specifically, IgTCR Signal 1 induces potent tumor-specific cytolytic functions, but all tumor-reactive T cell clones are eliminated during the response by AICD. In contrast, CD28-mediated Signal 2 (in combination with IgTCR stimulation) will induce tumor specific T cell proliferative functions in the presence of exogenous IL2, but is not sufficient to activate fully autonomous replication. Lastly, Signal 3 (LFA1) in combination with signals 1 and 2 completes the activation process by inducing enough endogenous IL2 release to allow T cells to replicate in a fully autonomous manner. The coordinated stimulation of all three receptors, and the activation of all three major response functions, is marked by the emergence of a subpopulation of T cells that are mature CD4/CD8 double positive cells. This distinct alteration in the subtypes of T cells present is due to the conversion of CD8-single positive cells to a CD4/CD8-double positive phenotype. CD4-single positive cells appear to remain as a distinct subtype.

"so what section"

The results achieved thus far indicate that we have made a significant advancement in understanding the key immune regulatory elements that will be necessary to reconstruct an effective anti-tumor immune response. T cells can penetrate virtually any body space and have the physical capability to destroy tumor cells. Immunotherapies have not been effective thus far because the means of achieving immune recognition of tumor cells and then inducing tumor specific effector functions have not been achieved. Our studies have provided crucial information on how to focus T cell immunity on tumor tissues. We have clearly shown that IgTCR molecules can direct T cells to recognize tumor tissues. We have also defined which receptor signals are required to activate each of the major T cell effector functions. Proof-of-principle studies indicate that the expression of these receptors, in a tumor-specific context allows the stepwise activation of the three major T cell effector functions. In principle, the efficient induction of the three major T cell response functions (killing, proliferation, IL2 release) should lead to an autonomous, self-sustaining immune response that will persist until all antigen-positive cells are eliminated. This principle is supported by our findings that combining tumor specific receptors induces T cells to behave in ways that are similar to what occurs during native immune responses. We plan to sequentially test the anti-tumor properties of T cells modified with combined receptors for Signal 1 plus Signal 2, and then Signals 1, 2 and 3 both in vitro and in vivo. We hypothesize that this comprehensive approach to activating anti-tumor immunity will ultimately lead to adoptive immunotherapies that are truly effective in eradicating malignant diseases.

10. REFERENCES

1. Kuwana, Y., Asakura, Y., Utsunomiya, N., Nakanishi, M., Arata, Y., Itoh, S., Nagase, F., and Kurosawa, Y. Expression of chimeric receptor composed of immunoglobulin-derived V regions and T cell receptor derived C regions. *Biochem. Biophys. Res. Commun. 1987:*149:960-968.

2. Gross, G., Waks, T., and Eshhar, Z. Expression of immunoglobulin-T-cells receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc. Natl. Acad. Sci. USA*. 1989:86:10024-10028.

3. Goverman, J., Gomez, S., Segesman, K., Hunkapiller, T., Laug, W., and Hood, L. Chimeric immunoglobulin-T cell receptor proteins form functional receptors: Implications for T cell receptor complex formation and activation. *Cell*. 1990:60:929-938.

4. Romeo, C., and Seed, B. Cellular immunity to HIV activated by CD4 fused to T cell or Fc receptor polypeptides. *Cell*. 1991:64:1037-1046.

5. Eshhar, Z., Waks, T., Gross, G., and Schindler, D. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the γ or ζ subunits of the immunoglobulin and T-cell receptors. *Proc. Natl. Acad. Sci. USA*. 1993:90:720-724.

6. Hwu, P., Shafer, G., Treisman, J., Schindler, D., Gross, G., Cowherd, R., Rosenberg, S., and Eshhar, Z. Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor γ chain. *J. Exper. Med.* 1993:178:361-366.

7. Roberts, M., Qin, L., Zhang, D., Smith, D., Tran, A-C., Dull, T., Groopman, J., Capon, D., Byrn, R., and Finer, M. Targeting of human immunodeficiency virus-infected cells by CD8+ T lymphocytes armed with universal T-cell receptors. *Blood*. 1994:84:2878-2889.

8. Becker, M.L.B., Near, R., Mudgett-Hunter, M., Margolies, M.N., Kubo, R., Kaye, J. and Hedrick S.M. Expression of a hybrid immunoglobulin-T cell receptor protein in transgenic mice. *Cell* 1989:58:911-921.

9. Moritz, D., Wells, W., Mattern, J., and Groner, B. Cytotoxic T lymphocytes with a grafted recognition specificity for ERBB2-expressing tumor cells. *Proc. Natl. Acad. Sci. USA*. 1994:91:4318-4322.

10. Weijtens, M.E.M., Willemsen, R., Valerio, D., Stam, K., and Bolhuis, R. Single chain Ig/γ gene-redirected human T lymphocytes produce cytokines, specifically lyse tumor cells, and recycle lytic capacity. *J. Immunol.* 1996:157:836-843.

11. Altenschmidt, U., Kahl, R., Moritz, D., Schnierle, S., Gerstmayer, B., Wels, W., and Groner, B. Cytolysis of tumor cells expressing the Neu/erbB-2, erbB-3, and erbB-4 receptors by genetically targeted naive T lymphocytes. *Clin. Can. Res.* 1996:2:1001-1008.

12. Yang, O., Tran, A-C., Kalams, S., Johnson, R., Roberts, M., and Walker, B. Lysis of HIV-I-infected cells and inhibition of viral replication by universal receptor T cells. *Proc. Natl. Acad. Sci. USA.* 1997:94:11478-11483.

13. Hombach, A., Heuser, C., Sircar, R., Tillmann, T., Diehl, V., Pohl, C., and Abken, H. An anti-CD30 chimeric receptor that mediates CD3-ζ-independent T-cell activation against Hodgkin's lymphoma cells in the presence of soluble CD30. *Can. Res.* 1998:58:1116-1119.

14. Beecham, E.J., Ortiz-Pujols, S., and Junghans, R.P. Dynamics of tumor cell killing by human T lymphocytes armed with an anti-CEA chimeric immunoglobulin T cell receptor. J. Immunother 23:332-343.

15. Beecham, E.J., Ripley, R, Ma, Q., and Junghans, R.P. Coupling CD28 costimulation to IgTCR molecules: dynamics of T cell proliferation and death. J. Immunother. Submitted.

16. Mcheyzer-Williams, M.G., and Davis, M.M. Antigen-specific development of primary and memory T cells in vivo. Science. 1995:268:106-111.

17. Bachmann, M.F., Speiser, D.E., and Ohashi, P.S., Functional maturation of and antiviral cytotoxic T cell response. J. Virol. 1997:71:5764-5768.

18. Busch, D.H., Pilip, I., and Pamer, E.G. Evolution of a complex TCR repertoire during primary and recall bacterial infection. J. Exp. Med. 1998:188:61-70.

19. Busch, D.H., and Pamer, E.G. T cell affinity maturation by selective expansion during infection. J. Exp. Med. 1999:189:701-709.

11. APPENDICES

A. Figures and Legends

B. Manuscripts

Beecham, E.J., Ortiz-Pujols, S., and Junghans, R.P. Dynamics of tumor cell killing by human T lymphocytes armed with an anti-CEA chimeric immunoglobulin T cell receptor. J. Immunother 23:332-343.

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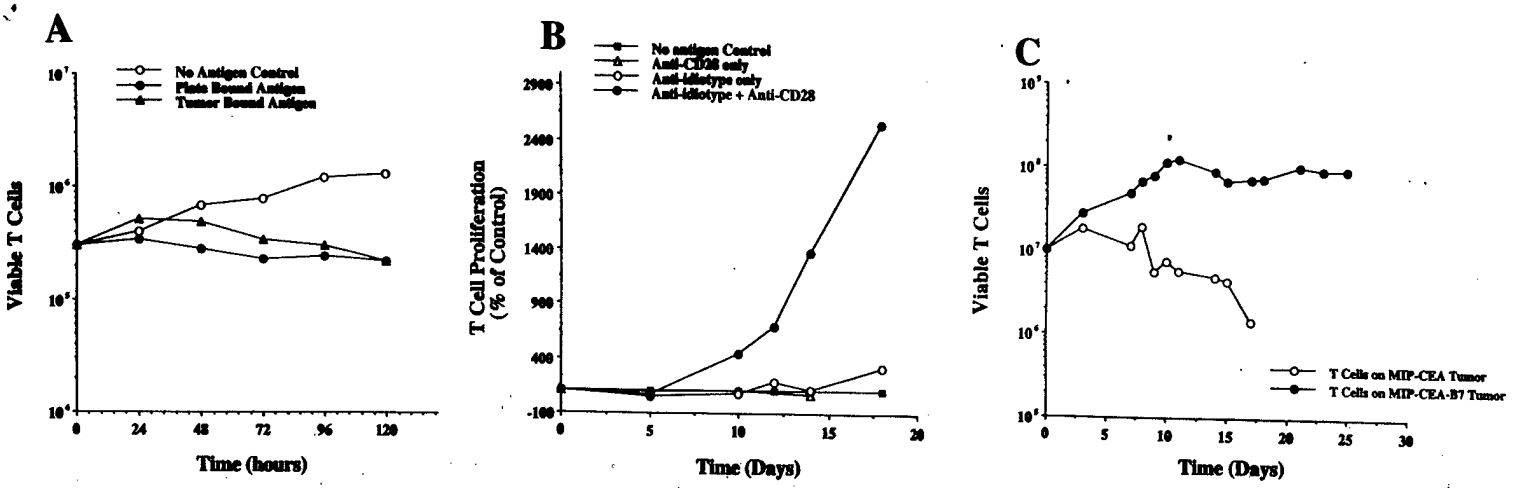


Fig. 1 ' T cell proliferation requires both Signal 1 and Signal 2. (A) Death of normal IgTCR-modified T cells incubated with either plate- or tumor-bound ligands/antigens compared to cells incubated with no antigenic stimulation. (B) Normal IgTCR-modified T cells incubated in tissue culture plates coated with different Abs as indicated in legend. Data from separate experiments normalized to the same T cells cultured in IL2-containing media with no bound Abs. (C) Death of normal IgTCR+ T cells incubated with tumor cells displaying only IgTCR-ligand, CEA (Signal 1; open circles), versus growth of the same T cells on tumor cells displaying IgTCR and CD28 ligands, CEA and B7, respectively (Signal 1 + 2; closed circles).

Fig. 2. Sustained T cell expansion with CD28 co-stimulation yields increased net tumor cell killing. (A) Death of IgTCR+ T cells incubated with Ag+ tumor cells without *B7 (Signal 1 only; open circles), and (B) growth of T cells on tumor* with *transduced B7 (Signal 1 + 2; closed circles). The curves of (A) are separately reproduced for Signal 1 (B) and for Signal 1 + 2 (C), with superposition of Ag+ tumor cells (triangles) that were "fed" to the T cells as stimulators.*

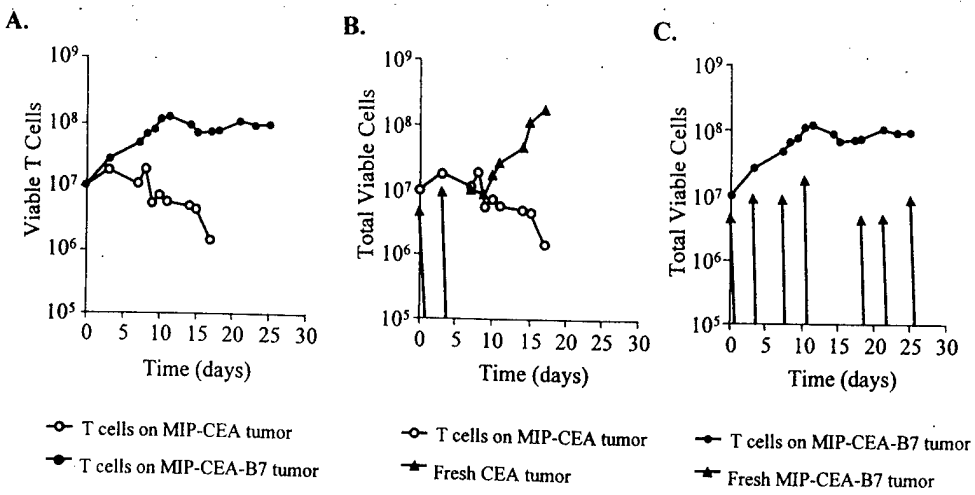
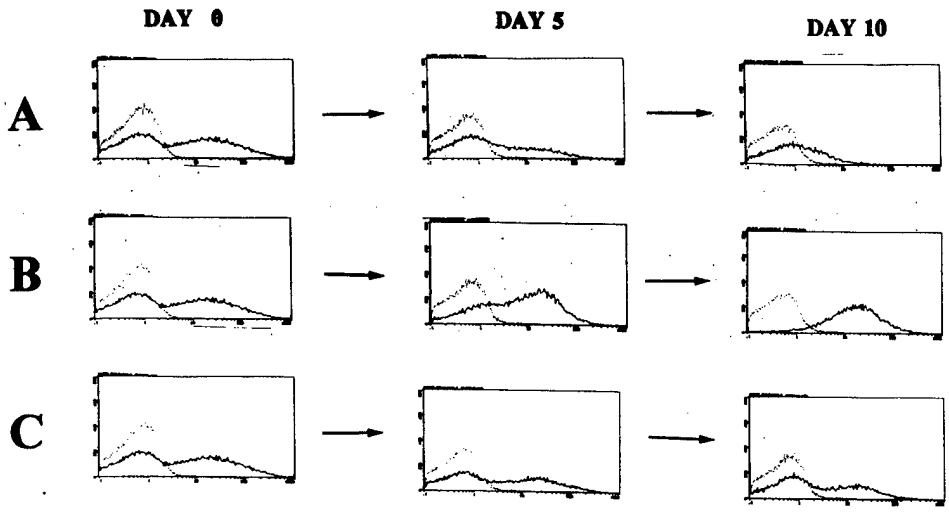


Fig. 3. Absence or presence of CD28 costimulation determines if T cells enter pathway of clonal deletion (AICD), or clonal expansion. A population of 50% IgTCR+ and 50% IgTCR− T cells (day 0) were stimulated with (A) Signal 1 (anti-IgTCR-only), (B) Signal 1+2 (anti-IgTCR +anti-CD28) or (C) were unstimulated. All cultures +IL2. IgTCR expression was assayed by FACS with anti-id (solid line) or negative control Ab (dotted line). Changes in relative proportions of IgTCR+ and IgTCR− T cells indicate selective deletion or expansion of IgTCR+ T cells.



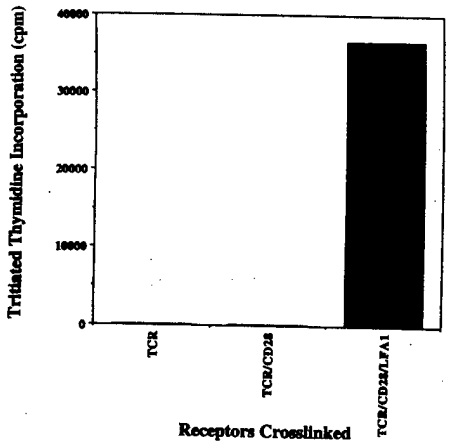
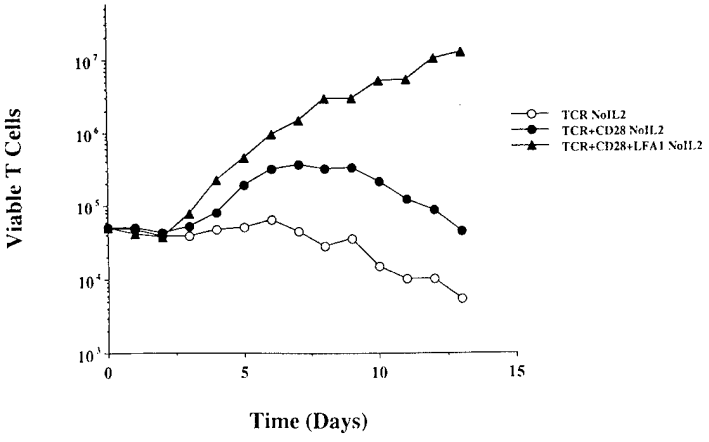


Fig. 4. Three signals are needed to induce IL2 release. Normal, unstimulated T cells were incubated in tissue culture plates coated with combinations of Abs to TCR, CD28, and LFA-1, without added IL2. Only the combination of 3 signals gave IL2 production. [IL2]>20 IU/ml; results of dilutions pending.

Fig. 5. Three signals are sufficient to support autonomous T cell proliferation. Cultures were activated with immobilized Abs to TCR, CD28 and/or LFA-1 and maintained in the absence of IL2. Resting T cells from a PBMC preparation were reduced in monocytes by one cycle of plastic adherence, which provide modest initial B7-mediated co-stimulation, but added monocytes do not reverse the −IL2 abortive 2-Signal profile. Days 5-8 were sampled and assayed for IL2 activity, as shown in Fig. 12.



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Dynamics of Tumor Cell Killing by Human T Lymphocytes Armed With an Anti-Carcinoembryonic Antigen Chimeric Immunoglobulin T-Cell Receptor

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Summary: Chimeric immunoglobulin T-cell receptors (IgTCR) join the antigen-binding portion of an antibody to one of the signaling chains of the TCR. A previous report described the construction and functional testing of an IgTCR gene directed against the carcinoembryonic tumor antigen (CEA). These preclinical studies showed the proper assembly and cell surface expression of anti-CEA IgTCR molecules, specific target antigen binding, and activation of T-cell effector functions. Although IgTCR-modified T cells function well in vitro, therapeutic applications in humans may be complicated by various factors, such as the availability of appropriate T-cell cytokines, high systemic levels of antagonistic soluble CEA, and antigenic diversity in tumor cell populations. The current study analyzes tumor cell killing by IgTCR-modified human T cells under conditions that more closely model those that may be encountered in persons with cancer. This analysis shows that 1) depriving IgTCR-modified T cells of interleukin-2 does not diminish anti-CEA cytotoxic T lymphocyte activity, but does eliminate killing by lymphokine-activated killer cells; 2) high levels of soluble CEA do not significantly inhibit tumor cell killing even when approximately 80% of the chimeric receptors are blocked; and 3) CEA^+ tumor cells that can down-regulate cell surface CEA evade immune destruction by IgTCR-modified T cells. These results have important implications for application strategies and protocol design considerations for early clinical testing of IgTCR anti-tumor therapies. **Key Words:** Immunoglobulin T-cell receptor—Cancer gene therapy—Adoptive immunotherapy—Carcinoembryonic antigen—Cytotoxic T lymphocyte—Lymphokine-activated killer cell.

Adoptive cellular immunotherapy for cancer treatment involves the transfer of cultured immune cells such as lymphokine-activated killer (LAK) cells or tumor-infiltrating lymphocytes to patients. Although LAK and tumor-infiltrating lymphocyte therapies have yielded some therapeutic responses, primarily in melanoma and renal cell cancer (1), their overall utility has been limited by a lack of tumor specificity by LAKs, and by the difficulty in isolating tumor-infiltrating lymphocytes that are not tolerant of the tumor. To overcome these problems, investigators have tried to augment cellular immunity by exploiting the high specificity of monoclonal antibodies. Initially, bispecific antibodies were conceived as a means to cross-link specific antigens on tumor cells with activation molecules on T or natural killer cells. However, this approach depends on the diffusion and binding interactions between three separate species (e.g., the tumor cell, bispecific antibody, and T cell), which is often problematic in vivo, particularly in the case of solid tumors. A more recent evolution of this concept has been to incorporate the antibody molecule

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directly into the T-cell receptor (TCR) through the construction of chimeric immunoglobulin TCRs (IgTCRs). Chimeric IgTCRs consist of the binding portion of an antibody, either as an Fab or sFv fragment, fused to one of the resident chains of the TCR complex.

Several laboratories have examined the structure and function of IgTCR molecules in vitro (2–14). These studies have shown that activation of T cells through the IgTCR molecule leads to the release of cytokines such as interleukin-2 (IL-2) (3,4,6,8,14), interferon (10), granulocyte-macrophage colony-stimulating factor (7,11), and tumor necrosis factor-α (3,11). Most of these studies have shown that IgTCR-modified effector cells are capable of redirected, antigen-specific cytolysis of target cells (4–14). Two studies (8,9) have shown that gene-modified T cells will proliferate in response to activation signals delivered through the IgTCR molecule. In vivo studies have been limited but have established the anti-tumor activity of IgTCR-modified effector cells in tumor-bearing animals (10,15).

The use of IgTCR-modified T cells in cancer treatment has several advantages and some possible disadvantages. One advantage is that the chimeric receptor molecule circumvents the development of tolerance by providing T cells with a receptor molecule that can specifically recognize and bind to a given tumor antigen. A second advantage is that the chimeric receptor allows T-cell activation to occur through a direct interaction with the tumor cell. This eliminates the need for antigen to be presented by accessory cells and allows IgTCR-modified T cells to activate their effector functions independently of major histocompatability complex (MHC) class I or II antigen presentation. Finally, activation through the chimeric receptor occurs regardless of the context of MHC class I expression. This removes the ability of tumor cells to avoid immune recognition by downregulating the expression of MHC class I molecules (16).

One potential drawback to the use of T cells armed with chimeric IgTCR genes is that circumventing the normal T-cell activation pathway may also bypass the recruitment of immune helper functions provided by various accessory cell populations. Thus, the normal system of "help" and cytokine expression that supports a naturally occurring immune response may be unavailable to IgTCR-modified cells infused into patients. The availability of IL-2 is crucial for the growth and viability of IgTCR-modified T cells. The secretion of IL-2 after antigen binding has been shown in transformed T-cell lines such as Jurkat cells (8,17), the MD45 mouse hybridoma line (3,6,14), and mouse EL4 cells (4). However, no study has shown that IgTCR$^+$ T cells derived from normal blood will secrete IL-2 after binding the target antigen. In the absence of exogenously added IL-2, IgTCR-modified T cells may lack anti-tumor activity.

A second potential drawback is that soluble forms of the targeted tumor antigen will also bind to the chimeric receptor, and these soluble ligand molecules may act as an antagonist. The concentration of soluble tumor antigen can reach very high levels in patients with cancer, and it is unknown what effect this will have on the gene-modified T cells or on their anti-tumor efficacy in vivo. Finally, any variant clones of the tumor that do not express the targeted tumor antigen will be able to evade immune destruction by IgTCR-modified T cells. It is likely that all of these problem areas will need to be overcome before IgTCR-modified T cells can be truly effective anti-cancer agents in humans.

We previously described (17,18) the construction and functional testing of several different IgTCR molecules directed against the carcinoembryonic tumor antigen (CEA). We showed the proper assembly and cell surface expression of anti-CEA IgTCR molecules, specific target antigen binding, and activation of T-cell effector functions after tumor cell binding (IL-2 release and tumor-specific cytolysis). In the current study, we have made a more systematic analysis of tumor cell killing by IgTCR-modified human T cells under conditions that more closely model those that we expect to encounter in persons with cancer. Specifically, we determined 1) the effect of IL-2 deprivation on tumor cell killing, 2) the relative resistance of anti-tumor activity to high levels of soluble CEA, and 3) the potential of antigen-negative variant tumor cells to evade immune destruction. The results are discussed in the context of application strategies for clinical testing of IgTCR anti-tumor therapies.

MATERIALS AND METHODS

Retroviral Vector and Vector Producer Cells

The construction of anti-CEA IgTCR genes has been described before (17). Based on equivalency in human T-cell activation tests and ease of expression, we selected a construct containing an sFv antibody fragment fused to the chain of the TCR for clinical development. This expression cassette contains sequences encoding the heavy and light chain variable regions (joined by a flexible linker) from the humanized MN14 antibody (17) fused to sequences encoding the ζ-chain of the human TCR. The antibody and ζ-chain sequences are separated by the hinge region of CD8α. The clinical retroviral vector was constructed by subcloning the 1.4-kb anti-CEAsFv expression cassette into the NcoI-BamHI sites of the MFG

vector backbone (provided by Dr. Richard Mulligan, Harvard Medical School, Boston, MA, U.S.A.). The transgene cassette is inserted such that the initiation codon of the inserted sequences is placed precisely at the position of the viral env initiation codon. The retroviral vector was designed to contain no selectable marker and no internal regulatory elements. Retroviral vector producer cells were constructed by transfecting the vector into the GP-E86 ecotropic helper cell line and using the transient viral supernatant to infect PG-13 cells. Viral supernatant from PG-13 cells was then used to transduce normal human peripheral blood lymphocytes.

Antibodies and Flow Cytometric Analysis

The humanized MN14 antibody and its anti-idiotype antibody, WI2 (19), were obtained from Immunomedics (Morris Plains, NJ, U.S.A.). The hMN14 was used in the construction of the chimeric receptor and to detect CEA expression on tumor cells. The WI2 was used to detect expression of the anti-CEA IgTCR construct, to select for anti-CEA IgTCR$^+$ T cells, and as a binding analog of CEA in experiments using plate-bound antigen. OKT3 (Ortho, Biotech, Raritan, NJ, U.S.A.) is a mouse antibody directed against the normal human TCR and was used as a positive control staining antibody and to activate human T cells. UPC-10 (Sigma Chemical, St. Louis, MO, U.S.A.) is an IgG2α mouse antibody with binding specificity for β-2-6–linked fructosan. UPC-10 was used as a negative control staining antibody in experiments using mouse antibodies. HAT (Hoffmann-La Roche, Nutley, NJ, U.S.A.) is a humanized IgG1,κ anti-Tac antibody that was used as a negative control staining antibody for experiments using hMN14. Fluorescein isothiocyanate (FITC)- and P-phycoerythrin (PE)–labeled antibodies against different human T-cell antigens (CD4, CD8, and CD16) and against mouse and human Fc were obtained from Caltag Laboratories (Burlingame, CA, U.S.A.). All antibody staining reactions were performed using standard methods (20). Fluorescence intensity was measured using a Coulter EPICS Profile II flow cytometer (Hialeah, FL, U.S.A.).

Cell Lines and Culture Conditions

MIP-101 is a poorly differentiated human colorectal cancer cell line that does not express CEA (21). The MIP-CEA cell line was derived by transfecting MIP-101 with a full-length cDNA encoding the human CEA gene (22). Both tumor cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin sulfate, and 2 mmol/L L-glutamine.

Lymphocyte Transduction and Culture

Peripheral blood mononuclear cells from normal blood were isolated by centrifugation over Histopaque-1083 (Sigma Chemical). T cells were activated by culturing cells for 48 hours in AIMV media (Gibco, Gaithersburg, MD, U.S.A.) supplemented with 100 U/mL IL-2 and 20 ng/mL OKT3. Activated T cells were transduced in anti-CEA IgTCR retroviral supernatant containing 10 μg/mL protamine sulfate (Fujisawa USA, Deerfield, IL, U.S.A.) centrifuged at 1,050*g* for 1 hour at 32°C. Peripheral blood mononuclear cells were transduced a total of two or three times. Although cell types other than T cells may be transduced at this stage, these contaminating cells are not stimulated to replicate under the culture conditions used, whereas treatment with OKT3 induces rapid T-cell proliferation. This selective T-cell proliferation quickly leads to cultures that are virtually 100% T cell in origin and effectively eliminates the influence of any contaminating cells from subsequent assays.

After transduction, cells were cultured for 2 days and then selected for anti-CEA IgTCR expression by binding anti-CEA IgTCR$^+$ cells to tissue culture plates coated with anti-idiotype antibody (positive panning) at either 37°C or 4°C for 30 minutes. Plates were coated by overnight incubation at room temperature with 0.1 mol/L sodium bicarbonate buffer containing 5 μg/mL antibody. Unbound T cells were removed from the plates by gently washing the plates two or three times with fresh media. Adherent cells were collected, expanded in T-cell growth media, and stained with PE-labeled WI2 and either fluorescein isothiocyanate–labeled mouse anti-human CD4 or mouse anti-human CD8 antibodies. The percentages of anti-CEA IgTCR–modified cells and the CD4:CD8 ratio were determined by flow cytometric analysis.

Cytotoxicity Assays

Tumor cell targets MIP-CEA (CEA positive) and MIP-101 (CEA negative) were plated into six-well tissue culture plates at a density of 1×10^5 cells/mL. After 24 hours, T cells transduced with the anti-CEA IgTCR vector were added to the plates at specific effector-to-target cell ratios (E:T ratio) and the plates were returned to incubation at 37°C. Every 24 hours, one well from each of the different E:T ratios was harvested for counting by first washing to remove unbound T cells, adding 0.5 mL trypsin to each well to detach the tumor

cells, adding 0.5 mL media (1 mL final volume), and then pipetting the final volume to create single-cell suspensions. Cells were diluted in media containing trypan blue, and the number of viable cells were counted using a hemocytometer.

Both MIP-CEA and MIP-101 cells are two to three times larger than human T cells and are readily distinguished from the few residual T cells that had not been removed by the pretrypsinization washes. The wash volumes used to remove unbound T cells were microscopically inspected to ensure that live tumor cells were not also detached from the plates. Although most cells in the wash volumes were either T cells or dead tumor cells, a very small number of live tumor cells could be detected. Because both tumor lines are strongly adherent, these cells most likely arise from the small fraction of cells undergoing mitotic detachment during washing. Because of their small numbers, live tumor cells contained in the wash volumes were not included in the cell counts. This was validated in our initial experiments by including live tumor cells from the wash volumes in the total cell counts and showing that either including or excluding the wash volume cell counts did not significantly alter the slope of the kill curves (data not shown). In subsequent experiments, wells that were to be harvested for counting were only microscopically inspected to ensure that significant numbers of viable, detached tumor cells were not present. Invariably, the only wells in which noticeable numbers of viable, detached tumor cells were observed was after day 4 or 5 in wells where little or no tumor cell killing occurred (e.g., untreated controls and MIP-101 cells). This occurred mainly because, in these wells, tumor cells proliferate unabated and become heavily confluent by the fourth and fifth days of the assay. Even in these cases, however, the exclusion of floating cells from the final cell counts had little effect on the overall slope of the growth curves (for example, see the slight decrease in slope of untreated controls from day 4 to day 5 in Figure 3).

For all cytotoxicity assays, individual wells were counted from two to nine times, depending on the cell density. For wells that contained a high density of cells, a minimum of two duplicate counts were performed. When extensive cell death made it difficult to obtain more than 100 cells for counting, a minimum of nine counts were done. These cell counts were averaged to obtain the total number of cells remaining for each time and treatment group in a given experiment. Cell counts from repeated independent experiments were averaged for each time and treatment group and the number of surviving tumor cells was plotted as a function of time.

RESULTS

Stable, Long-Term Expression of the IgTCR Vector in Normal Human T Cells

Cultures of primary human T lymphocytes were transduced with the anti-CEA IgTCR vector and assayed for IgTCR expression. After two or three rounds of transduction, approximately 40% to 50% of the T cells express the anti-CEA IgTCR gene (Fig. 1A). Cells that did not bind to the anti-idiotype coated plates did not express the anti-CEA IgTCR protein (Fig. 1B). Gene-modified T cells were selected by binding the cells to tissue culture plates coated with anti-idiotype antibody (Fig. 1C). 100% of the panned cells could rebind anti-idiotype–coated plates (Fig. 1D), indicating that the low-staining fraction of cells in Figure 1C express physiologically relevant levels of the chimeric protein. T cells that were bound a second time to anti-idiotype–coated plates (Fig. 1D) showed a similar pattern of staining as those that were bound only once. This indicates that additional rounds of panning at 37°C did not further increase the level of anti-CEA IgTCR expression (compare Figures 1C and 1D), and that one round of panning fully selects for anti-CEA IgTCR+ T cells.

The level of anti-CEA IgTCR expression in the selected population of cells was sensitive to temperature. Panning cells at 4°C failed to bind low-expressing cells, resulting in a selection bias for cells that expressed higher levels of anti-CEA IgTCR protein (compare Figures 1C and 1E). At 4°C, the T cells assume a uniformly round shape with reduced membrane fluidity. This rounded shape presents a reduced area of the membrane to the plate surface. Cells that express quantitatively higher densities of receptors per square micron have a selective advantage in remaining bound to the plates during washes. Thus, panning at 4°C yields populations of cells with higher levels of IgTCR expression. At 37°C, the cells have normal membrane fluidity and become progressively flattened on the bottom of the plates as more receptors surrounding the initial contact point bind immobilized antigen. This large area of binding facilitates the engagement of sufficient receptors on cells with low densities of receptors to allow them to remain bound to the plates during washes.

Both CD4+ and CD8+ T Cells Are Modified With the Anti-Carcinoembryonic Antigen IgTCR Gene

T cells from Figure 1C were stained with antibodies against the CD4 or CD8 receptors, and either WI2-PE or UPC-PE, and then examined by two-color flow cytomet-

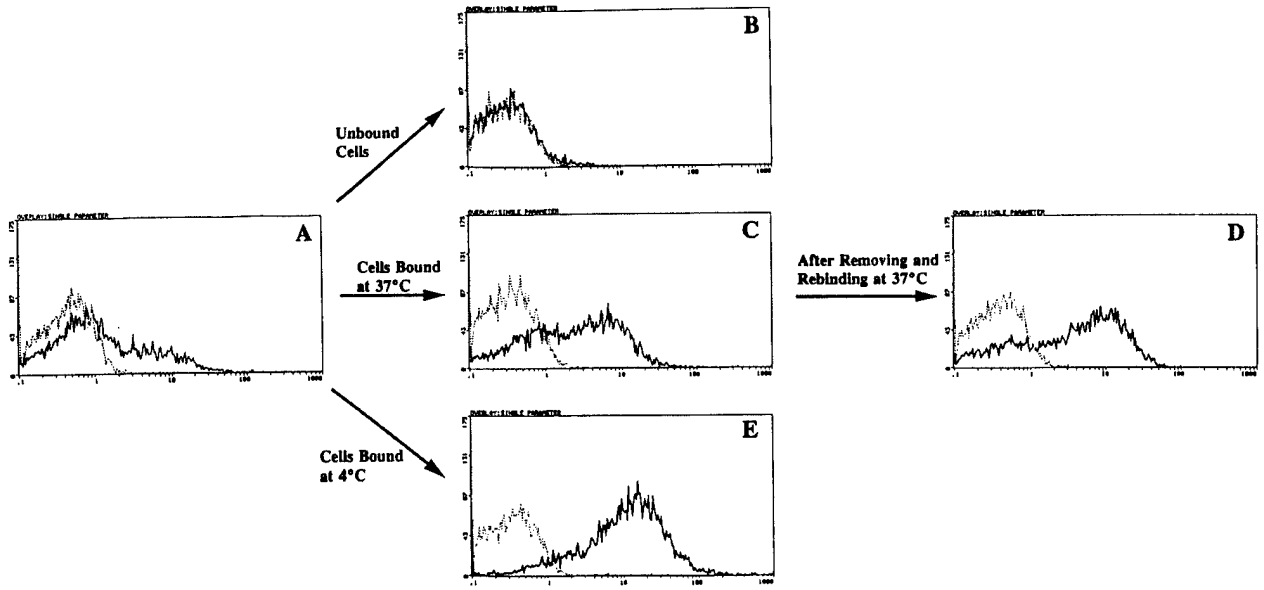

FIG. 1. Normal human T lymphocytes transduced with the anti-CEA IgTCR vector. OKT3-activated human T cells were infected twice with the anti-CEA IgTCR vector, stained with either PE-labeled WI2 anti-idiotype antibody (solid lines) or with UPC negative control antibody (dotted lines), and then examined by flow cytometric analysis. **A:** T cells stained with WI2 or UPC 1 day after the second round of infection. **B:** T cells from panel A that did not bind to anti-idiotype–coated tissue culture plates. **C:** T cells from panel A selected for anti-CEA IgTCR expression by binding the cells to anti-idiotype–coated tissue culture plates at 37°C. **D:** T cells from panel C that rebound anti-idiotype–coated tissue culture plates at 37°C. **E:** T cells from panel A selected for anti-CEA IgTCR expression by binding the cells to anti-idiotype–coated tissue culture plates at 4°C.

ric analysis (Fig. 2). $CD4^+$ and $CD8^+$ T cells were transduced at roughly equal proportions with the IgTCR vector.

Expression of the anti-CEA IgTCR gene was stable in long-term cultures of primary human T cells, remaining virtually unchanged over a period of more than 2 months (data not shown). The ratio of $CD4^+$ to $CD8^+$ T cells was also stable over time, although in cultures carried for more than 2 months the proportion of $CD8^+$ cells slowly increased (data not shown). All T-cell cytotoxicity experiments described in this report were performed using populations of T cells that were 100% positive for expression of the anti-CEA IgTCR gene and contained CD4:CD8 ratios of approximately 60:40. Populations of T cells were selected for anti-CEA IgTCR expression by anti-idiotype panning at 37°C, as noted before.

Potent, Targeted Killing of Carcinoembryonic Antigen-Positive Tumor Cells

In the current study, tumor cell killing was measured using a trypan-blue–based assay. This assay allows tumor cell killing to be measured cumulatively during a period of several days, and thus it is more sensitive than ^{51}Cr release assays. This increased sensitivity allows the use of much lower E:T cell ratios. Compared with ^{51}Cr release assays, this assay has a more directly interpret-

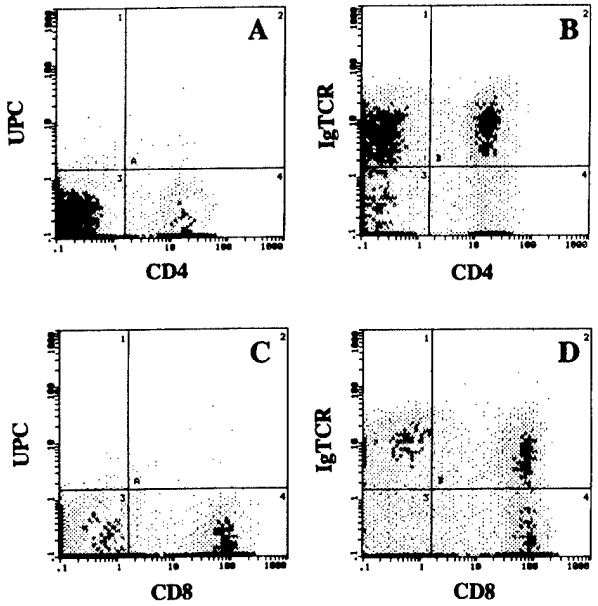

FIG. 2. IgTCR modification of both $CD4^+$ and $CD8^+$ T cells. The proportion of $CD4^+$, $CD8^+$, and $IgTCR^+$ T cells was determined by two-color flow cytometric analysis. Green fluorescence (fluorescein isothiocyanate channel [FITC]) is plotted on the x axis and red fluorescence (PE channel) is plotted on the y axis. T cells from Figure 1C were stained with the following antibodies. **A:** Mouse-anti-human CD4-FITC and negative control antibody UPC-PE. **B:** Mouse-anti-human CD4-FITC and WI2 antidiotype anitbody. **C:** Mouse-anti-human CD8-FITC and negative control antibody UPC-PE. **D:** Mouse-anti-human CD8-FITC and WI2 antidiotype anitbody.

able meaning in terms of net tumor cell killing. A comparison of cytotoxicity data using both the trypan-blue–based and a ^{51}Cr release assay showed that the trypan blue assay had greater sensitivity, was more reproducible, and did not suffer from the high background counts associated with the leakage of ^{51}Cr from untreated cells (data not shown). Other validation studies are described in Materials and Methods.

Cytotoxicity at different E:T ratios was determined by coincubating anti-CEA IgTCR-modified T cells (from Fig. 1C) with either the CEA-negative cell line, MIP-101, or with CEA-positive MIP-CEA cells. Viable tumor cells in each treatment group were counted by trypan blue exclusion and plotted as a function of incubation time (Fig. 3). At an E:T ratio of 0.5:1, MIP-CEA cells were progressively destroyed during a period of several days (Fig. 3A). At higher E:T ratios, the elimination of MIP-CEA tumor cells was accelerated in a dose-dependent manner.

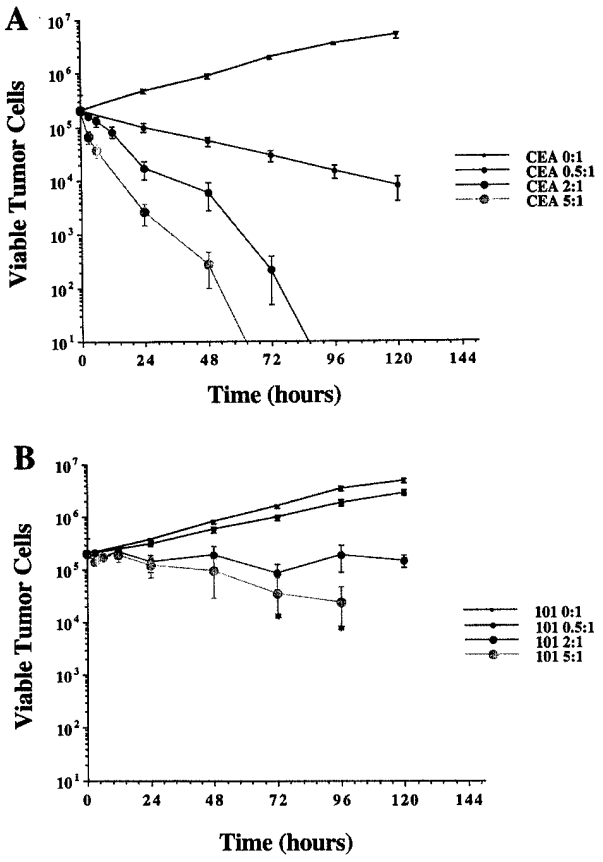

FIG. 3. Targeted killing of CEA-positive tumor cells. **A:** CEA-positive tumor cells (MIP-CEA) or **B:** CEA-negative tumor cells (MIP-101) were coincubated with anti-CEA IgTCR-modified T cells. Viable tumor cells are plotted as a function of time. Data were averaged from five to seven individual experiments at each of the E:T ratios shown in the figure legends. Error bars denote ± the standard deviation. *Error bars at 72 and 96 hours in the 5:1 E:T ratio are negative because of zero values in one of the repeated experiments, which cannot be graphed.

In contrast to MIP-CEA, MIP-101 cells continued to proliferate when incubated with anti-CEA IgTCR+ T cells at low E:T ratios (Fig. 3B), although their growth rate was reproducibly lower than untreated controls. Nonspecific killing of MIP-101 cells occurred at all E:T ratios above 2:1. Although this toxicity was significantly less than that against MIP-CEA cells, it nonetheless occurred in a dose-dependent manner. Depletion of any residual natural killer cells from the T-cell cultures by negative panning with anti-CD16 antibody did not eliminate this nonspecific cytotoxicity (data not shown).

T Cells Retain the Ability to Lyse Carcinoembryonic Antigen-Positive Tumor Cells in the Absence of Interleukin-2

A primary goal of the current studies was to examine anti-CEA IgTCR-mediated cytolysis under conditions similar to those that might occur in vivo in human clinical trials. The availability of IL-2 in vivo could play a significant role in supporting the ability of IgTCR-modified T cells to activate anti-tumor effector functions. IL-2 and other cytokines are secreted by CD4+ gene-modified cells in response to binding target antigen (3,4,6–11,14,17). However, it is uncertain whether local secretion will be sufficient to maintain T-cell viability and activity. To determine the importance of the continued presence of IL-2, we examined the effect of IL-2 withdrawal on anti-CEA IgTCR-mediated cytotoxicity.

T cells were prepared by culturing separate aliquots of cells from Figure 1C in media that contained either 100 U/mL IL-2 or no exogenously added IL-2. Approximately 70% of the T cells die within 7 to 10 days after IL-2 is removed from the cultures, as shown by the high proportion of cells with low forward scatter (Fig. 4A). Dead cells were removed from the cultures by centrifugation over Histopaque-1083 and the remaining live cells were used in tumor cell killing assays. In IL-2–deprived cultures, the surviving T cells changed from an activated appearance (large, irregularly shaped cells) to a resting appearance (small, homogeneously round cells) (Fig. 4A). In addition, the expression of the anti-CEA IgTCR gene decreased significantly in IL-2–rested cultures (Fig. 4B), paralleling the generalized downregulation of protein synthesis, including actin and normal TCR proteins (X. Y. Tan and R. P. Junghans, unpublished data). Despite these changes, IL-2–rested T cells retain their ability to kill MIP-CEA tumor cells (Fig. 5A). Further, the nonspecific toxicity previously observed against MIP-101 target cells was completely eliminated (Fig. 5B).

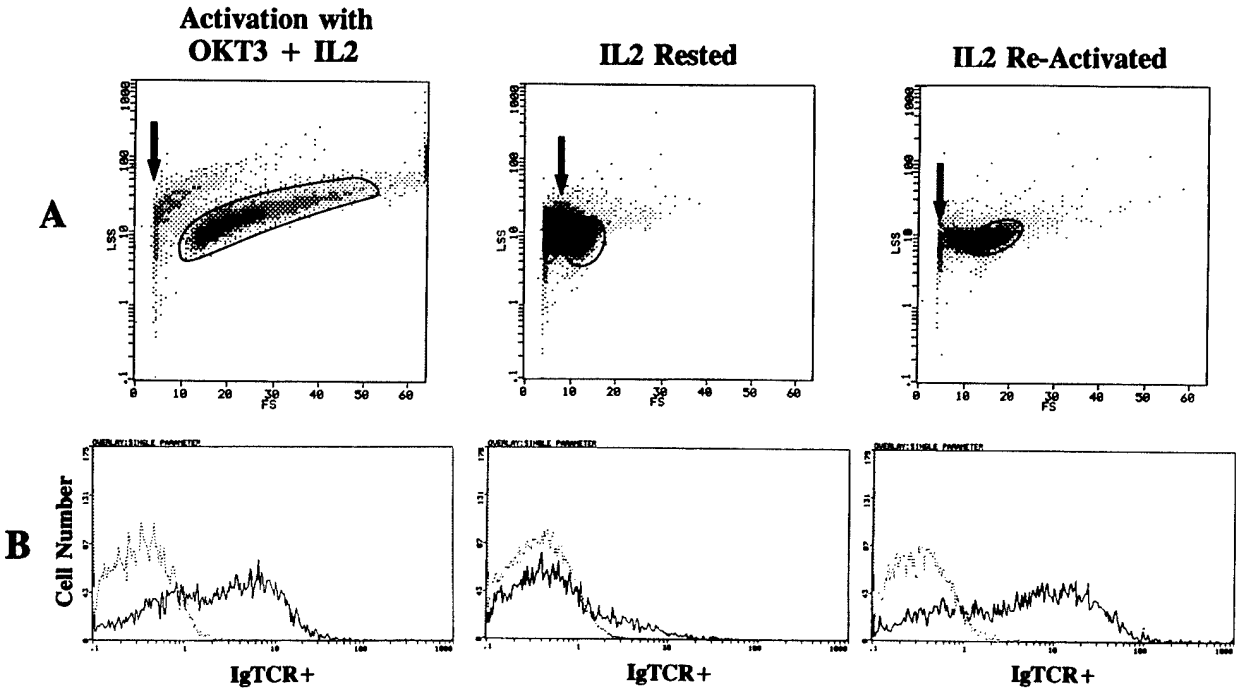

FIG. 4. IL-2–dependent T-cell morphologic changes. Flow cytometric analysis of human T cells after activation with OKT3 + IL-2, 10 days after removal of IL-2 from the cultures (IL-2 rested), and 2 or 3 days after cells were restimulated with IL-2 (IL-2 reactivated). **A:** Flow cytometric profiles of log side scatter (LSS) versus forward scatter. Populations of live cells are shown enclosed in the circles used to gate the cells on the flow cytometer. Populations of dead and dying cells are indicated with arrows. **B:** Fluorescence intensity of cells stained with anti-idiotype antibody ($IgTCR^+$).

Even at very high E:T ratios, where all MIP-CEA cells are lysed within 24 hours (Fig. 6A), MIP-101 target cells incubated with IL-2–rested T cells proliferate as rapidly as untreated controls (Fig. 6B). Control assays using $IgTCR^+$ T cells that remained in IL-2 media, and done in parallel with the experiments shown in Figures 5A and B, gave similar rates of killing at each E:T ratio (data not shown). Thus, differences in the absolute rate of killing between Figure 3, Figure 5, and Figure 6 are due to interassay variability.

As noted before, the removal of natural killer cells from the cultures by negative panning on CD16-coated plates did not eliminate nonspecific toxicity against MIP-101 cells, whereas removing IL-2 from the cultures did. Next we wanted to determine if the nonspecific toxicity against MIP-101 cells resulted from the activation state of the T cells or from the elimination of a specific subset of T cells that were selected against by IL-2 deprivation. T-cell cultures rested as in Figure 4 were returned to IL-2–containing media for 2 or 3 days. These IL-2–rescued T cells recovered their activated appearance (Fig. 4A) and high anti-CEA IgTCR expression (Fig. 4B). Nonspecific cytotoxicity also reappeared in these IL-2–rescued T cells (Compare Figs. 6B and 5C). Subsequent experiments showed that removal of IL-2 from the cultures 1 hour before the coincubation step was sufficient to eliminate nonspecific cytotoxicity to MIP-101 (data not shown). No T-cell death occurs after only 1 hour of IL-2 deprivation.

High Levels of Soluble Carcinoembryonic Antigen Do Not Prevent Anti-Carcinoembryonic Antigen IgTCR-Mediated Cytotoxicity

An important question is what effect high concentrations of soluble CEA will have on the kinetics of anti-CEA IgTCR-mediated tumor cell killing. To determine the effect of soluble CEA, T cells were incubated with MIP-CEA cells in the presence of 1,000 and 10,000 ng/mL soluble CEA (Figs. 7A and B, respectively,). Anti-CEA IgTCR-modified T cells efficiently killed MIP-CEA tumor cells in the presence of both 1,000 and 10,000 ng/mL soluble CEA. In some of the kill curves, the rate of cytolysis was slightly slower in the presence of soluble CEA than in its absence, but even in these instances soluble CEA did not substantially block tumor cell killing.

Immune Evasion by Tumor Cells That Have Downregulated Cell Surface Carcinoembryonic Antigen

At very low E:T ratios (<0.1 E:T), many CEA-positive tumor cells are killed by anti-CEA IgTCR-modified

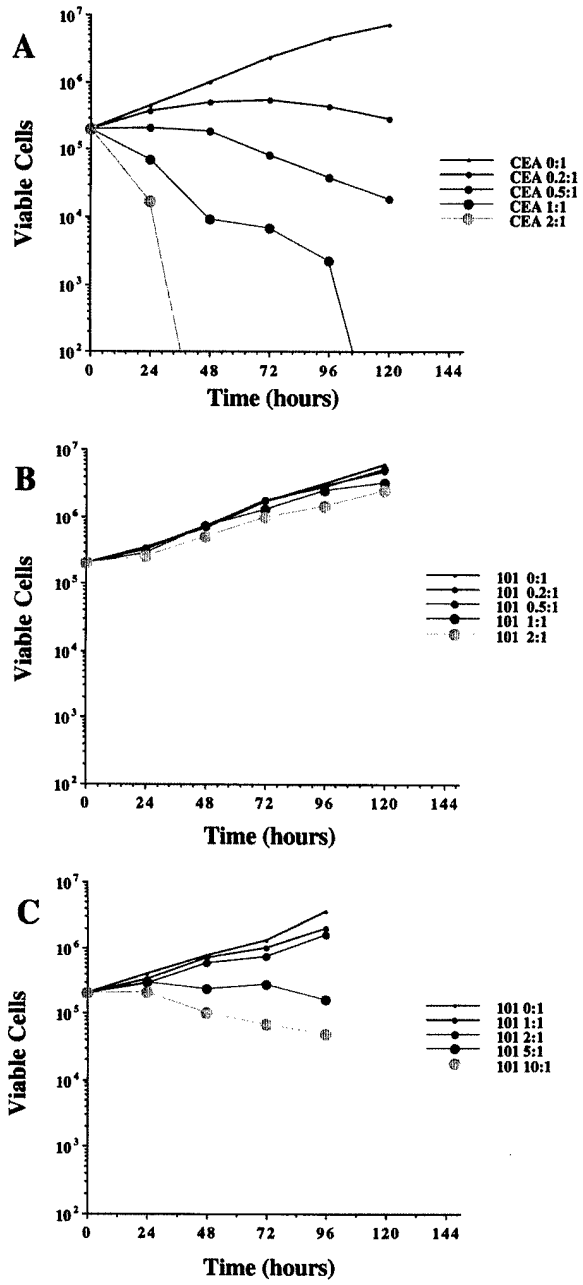

FIG. 5. Tumor cell killing in the absence of exogenous IL-2. Anti-CEA IgTCR-modified T cells were IL-2 rested for 7 to 10 days, and dead cells were removed by centrifugation over ficoll. IL-2–rested T cells were coincubated with MIP-CEA and MIP-101 tumor cells. **A:** MIP-CEA tumor cell killing in the absence of IL-2. **B:** MIP-101 tumor cell killing in the absence of IL-2. **C:** MIP-101 tumor cell killing by T cells cultured without IL-2 for 10 days and then reactivated by culturing the cells in IL-2–containing media for 2 or 3 days. The E:T ratios used in each experiment are shown next to each graph. The E:T ratios used in panel C are greater than those in panels A and B to detect any nonspecific toxicity.

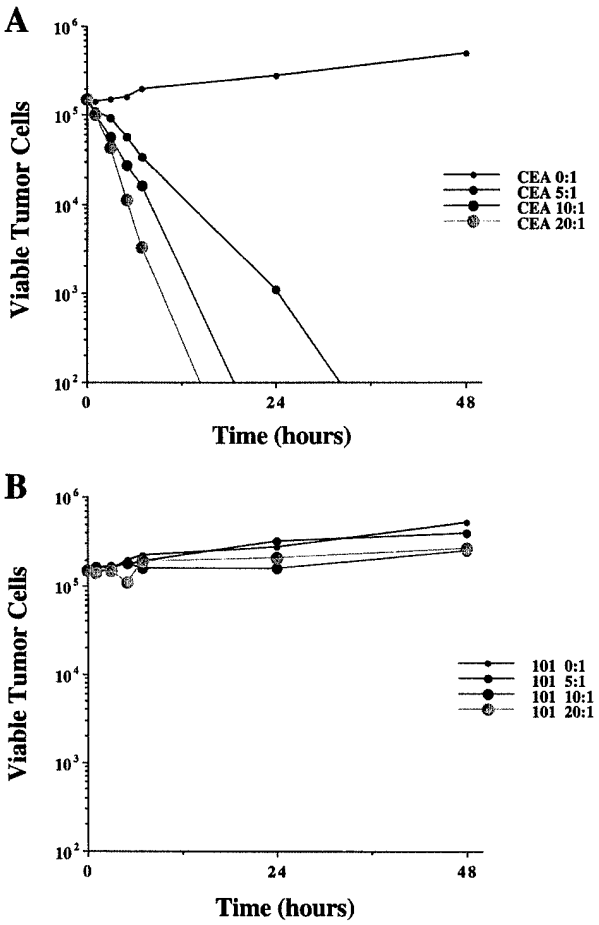

FIG. 6. Complete loss of T-LAK activity with IL-2–rested T cells. IL-2–rested anti-CEA IgTCR modified T cells, as in Figure 4, were coincubated with MIP-CEA and MIP-101 tumor cells and cytoxicity was measured. **A:** MIP-CEA tumor cell killing in the absence of IL-2. **B:** MIP-101 tumor cell killing in the absence of IL-2. The E:T ratios used in each experiment are shown next to each graph.

T cells. Despite this, a fraction of the tumor cells escape T-cell killing and eventually go on to overgrow the cultures (data not shown). These surviving tumor cells grow as distinct colonies, indicating that survival was the result of either random immune escape caused by the sparse seeding of T cells, or a specific selection of tumor cell variants that are resistant to anti-CEA killing. To determine if colony formation was due to specific evasion of anti-CEA T cells, surviving tumor cells were expanded in culture and then analyzed for cell surface CEA expression. These oligoclonal MIP-CEA survivors were stained with hMN14 antibody and their cell surface CEA levels were compared with those of MIP-CEA cells that had not been exposed to anti-CEA IgTCR T cells (Fig. 8). As expected, the parental MIP-101 and MIP-CEA tumor cells showed no deviation from their characteristic CEA expression profiles (Figs. 8A and B, re-

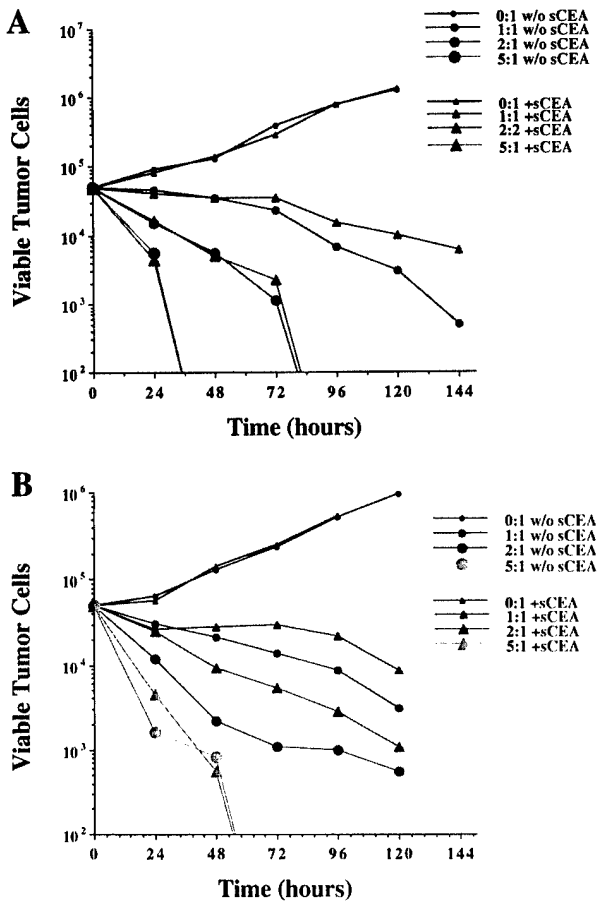

FIG. 7. Preservation of tumor cell killing in the presence of soluble CEA. Anti-CEA IgTCR-modified T cells were coincubated with MIP-CEA tumor cells in the presence and absence of soluble CEA. **A:** MIP-CEA tumor cell killing in the presence of 1,000 ng/mL soluble CEA. **B:** MIP-CEA tumor cell killing in the presence of 10,000 ng/mL soluble CEA. The E:T ratios used in each experiment are shown next to each graph.

spectively). However, the oligoclonal MIP-CEA survivors from the cytotoxicity assay (Fig. 8C) included a preponderance of cells with lower cell surface CEA protein. Most of these survivor cells have no detectable CEA, whereas a smaller fraction (≈20%) retain CEA expression that is slightly lower than the parental MIP-CEA cell line. This latter group may be a result of stochastic escape in which a fraction of CEA-positive tumor cells escape containment by the sparsely seeded T cells.

DISCUSSION

Expression of the IgTCR Vector in Normal Human T Cells

To facilitate long-term engraftment of the vector-modified cells, we designed the anti-CEA IgTCR vector to contain virtually no foreign gene products. This was accomplished by using a humanized antibody to derive the immunoglobulin portion of the chimeric molecule and human sequences for the hinge–TCR portions of the chimera (17). Furthermore, no microbial drug selection marker was included in the vector. To overcome the lack of drug selection, we substituted positive panning of transduced cells on anti-idiotype–coated plates to select gene-modified T cells. As shown in Figure 1, this method of selection can yield preparations of cells that are 100% positive for the transgene as rapidly as 30 minutes. An added advantage of this method is that se-

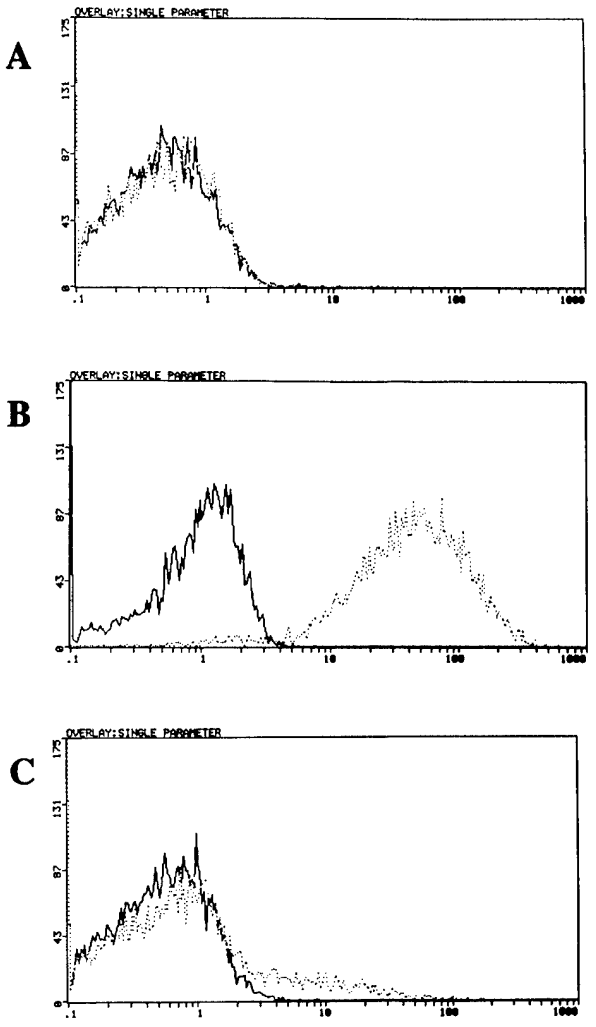

FIG. 8. CEA-positive tumor cells that lack cell surface CEA evade immune destruction. Tumor cells surviving in culture after coincubation with a low number of T cells (E:T, < 0.1:1) were analyzed for surface CEA expression. Tumor cells were stained with negative control antibody (solid line) or with hMN14 antibody (dotted line). **A:** MIP-101 tumor cells. **B:** Parental MIP-CEA tumor cells. **C:** MIP-CEA oligoclonal survivor cells from cytoxicity assays.

lection is based on expression of the therapeutic transgene itself rather than on an irrelevant marker gene.

Targeted Killing of Carcinoembryonic Antigen$^+$ Tumor Cells

From a therapeutic perspective, the most important functional test of anti-CEA IgTCR-modified T cells is their ability to redirect cytolytic functions and specifically lyse CEA-positive tumor cells. At an E:T ratio of 0.5:1, less than 4% of the CEA-negative tumor cells remained viable after 5 days, whereas CEA-positive tumor cells expanded approximately 15 times during the same period. Approximately 40% of these T cells are CD8$^+$ cells, which yields a cytotoxic T lymphocyte (CTL)-to-tumor cell ratio of 0.2:1. The elimination of tumor cells at this low effector-to-target cell ratio confirms that modified T cells recycle their lytic capacity to kill multiple targets, as in normal T-cell killing. Given the sparseness of the initial T-cell seeding (2×10^5 cells per well of a six-well plate), this also indicates that the modified T cells are mobile on the plate in finding the targets, which are themselves adherent. Similar experiments using peripheral blood mononuclear cells isolated from several patients with colon cancer showed no significant differences in CEA-directed cytolysis in comparison to peripheral blood mononuclear cells derived from healthy donors (data not shown). The independent roles of CD4$^+$ and CD8$^+$ T cells in tumor cell killing is being investigated.

Nonspecific killing of CEA-negative tumor cells depends on IL-2, suggesting that this toxicity was mediated by T-LAK cells (23,24), which are lymphokine-activated killer cells derived from T-cell precursors. T-LAK cells are characterized by their ability to lyse a wide spectrum of tumor targets. They can develop cytotoxic activity in the absence of any antigenic stimulation (23,24). Although there are conflicting interpretations of the origins of LAK precursors and their characteristic phenotypes as effector cells (25–29), it is clear that LAK cells can be derived from either natural killer or T-cell precursors.

Antigen-Specific Killing Is Preserved in the Absence of IL-2, but T-Lymphokine-Activated Killer Activity Is Eliminated

In vivo levels of IL-2 in cancer patients treated with IgTCR-modified T cells may play a critical role in regulating anti-tumor efficacy. Although CD4$^+$ IgTCR-modified T cells secrete IL-2 after binding antigen (17), it is not known if this is sufficient to support tumor cell killing in the absence of exogenously added IL-2. As such, we wanted to determine the effect of IL-2 deprivation on anti-tumor activity. As expected, depriving the T cells of IL-2 led to apoptosis and a marked decrease in viability. T cells that survived IL-2 withdrawal converted to a deactivated phenotype (Fig. 4). Despite this general deactivation, IL-2–rested T cells retained the ability to respond to CEA-positive tumor cells and were undiminished, on a per cell basis, in their ability to lyse CEA-positive tumor cells (Fig. 5). Similarly, the low levels of IgTCR protein expressed in IL-2–rested cells (Fig. 4B) did not significantly reduce CTL activity. This indicates that IgTCR expression that is below detection by fluorescence-activated cell sorting is nonetheless physiologically functional.

Depriving T cells of IL-2 also served to illuminate the two distinct mechanisms by which tumor cell killing is accomplished in this model. When exogenous IL-2 is withdrawn, tumor cell killing is exclusively CEA specific and is mediated through a CTL-like mechanism. Although IgTCR-mediated killing is not MHC dependent, as in normal CTL killing, it is antigen-dependent and uses a similar signaling process. Withdrawing IL-2 just before the coincubation step was sufficient to eliminate all nonspecific toxicity (data not shown), indicating that IL-2 is the key mediator of this nonspecific toxicity. Even at very high doses of T cells (Figs. 6A and B), tumor cytolysis is entirely antigen specific in the absence of IL-2, whereas in the presence of IL-2 there is a moderate level of nonspecific T-LAK activity against MIP-101 cells (data not shown).

In the presence of exogenous IL-2, tumor cell killing appears to occur through a combination of nonspecific LAK-like activity (T-LAK) and antigen-specific CTL-like activity. LAK cells lyse a wide spectrum of target cells independently of any antigenic stimulation (23,24), whereas CTLs only lyse cells that display the target antigen. The results show that nonspecific T-LAK–mediated killing is active only in the presence of IL-2 (Fig. 5C). Comparing the proportion of killing attributable to each type of mechanism shows that CTL-type killing is far more potent than LAK-type killing. For example, at an E:T ratio of 0.5:1, nonspecific LAK-type killing accounts for an approximately one- to two-fold reduction in tumor cell number (Fig. 3B), whereas antigen-specific CTL-type killing accounts for an approximately 500-fold reduction (Fig. 3A).

Soluble Carcinoembryonic Antigen Does Not Block Tumor Cell Killing

Many patients with CEA-expressing tumors have high serum levels of soluble CEA, which may inhibit anti-CEA IgTCR-mediated tumor cell killing. The level of soluble CEA in the serum of patients infrequently ex-

ceeds 1,000 ng/mL (18), whereas levels that are 10 times greater might be expected to occur in the immediate tumor bed microenvironment. Tumor cell killing was not blocked at either of these concentrations of soluble CEA, although there is a modest dose-dependent inhibition that is most readily detected at the lower E:T ratios.

Expressed in terms of kD, soluble CEA at 1,000 ng/mL (5 nmol/L) is well below the sFv kD of 21 nmol/L and will not significantly reduce the number of free chimeric receptors (17). Soluble CEA at 10,000 ng/mL (50 nmol/L) is approximately 2.5 times the sFv kD and will reduce the number of free receptor molecules by approximately 80%. Previously we hypothesized that soluble CEA, which can only bind monovalently to the chimeric receptor, will not be able to compete with the stabilized, polyvalent interactions that occur with immobilized CEA on the surface of tumor cells. The fact that cytotoxicity was only slightly reduced when approximately 80% of the receptors are blocked supports this hypothesis and suggests that soluble CEA will not be able to block cytotoxicity in vivo.

Immune Evasion by Tumor Cells that Have Downregulated Carcinoembryonic Antigen Surface Expression

It is becoming clear that tumor cells can evolve several mechanisms to evade immune destruction, including downregulating MHC class I expression, Fas ligand-induced apoptosis of infiltrating lymphocytes (30,31), and secreted immunosuppressive factors (e.g., TGFβ) (32). T cells redirected by chimeric IgTCR genes will not be affected by some of these mechanisms (e.g., MHC class I repression) but may be inhibited by others.

The simplest mechanism by which tumor cells can evade anti-CEA IgTCR-modified T cells is to downregulate expression of the target antigen. Most tumor cells that evaded anti-CEA IgTCR-mediated killing had no detectable CEA expression by flow cytometry, whereas a smaller fraction (approximately 20%) expressed low levels of CEA. Close examination of the CEA expression profile in the parental MIP-CEA cells (Fig. 8B) reveals a small tail of low- or nonexpressing tumor cells. These low expressing tumor cells are most likely the progenitors of CEA-negative surviving tumor cells in Fig. 8C. Surviving tumor cells that still express CEA may have escaped destruction by the sparse seeding and lack of contact during the assay period. In vivo, it is possible that some tumor cells will evade immune destruction by anti-CEA IgTCR-modified T cells simply by loss of CEA expression. Ultimately, it may be necessary to provide chimeric receptors to two or more tumor antigens. For example, T cells targeted to colon cancers could be armed with chimeric receptors to both the CEA and Tag-72 tumor antigens.

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REFERENCES

1. Cohen PA, Hwu P, Rosenberg SA. Adoptive cellular immunotherapy and gene therapy. In: Chabner BA, Longo DL, eds. *Cancer Chemotherapy and Biotherapy: Principles and Practice, 2nd ed.* Philadelphia: Lippincott-Raven Publishers, 1996, pp 721–47.
2. Kuwana Y, Asakura Y, Utsunomiya N, et al. Expression of chimeric receptor composed of immunoglobulin-derived V regions and T cell receptor derived C regions. *Biochem Biophys Res Commun* 1987;149:960–8.
3. Gross G, Waks T, Eshhar Z. Expression of immunoglobulin-T-cells receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc Natl Acad Sci USA* 1989;86:10024–8.
4. Goverman J, Gomez S, Segesman K, et al. Chimeric immunoglobulin-T cell receptor proteins form functional receptors: implications for T cell receptor complex formation and activation. *Cell* 1990;60:929–38.
5. Romeo C, Seed B. Cellular immunity to HIV activated by CD4 fused to T cell or Fc receptor polypeptides. *Cell* 1991;64:1037–46.
6. Eshhar Z, Waks T, Gross G, Schindler D. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the or subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci USA* 1993;90:720–4.
7. Hwu P, Shafer G, Treisman J, et al. Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor chain. *J Exp Med* 1993;178:361–6.
8. Roberts M, Qin L, Zhang D, et al. Targeting of human immunodeficiency virus-infected cells by $CD8^+$ T lymphocytes armed with universal T-cell receptors. *Blood* 1994;84:2878–89.
9. Becker MLB, Near R, Mudgett-Hunter M, et al. Expression of a hybrid immunoglobulin-T cell receptor protein in transgenic mice. *Cell* 1989;58:911–21.
10. Moritz D, Wells W, Mattern J, Groner B. Cytotoxic T lymphocytes with a grafted recognition specificity for ERBB2-expressing tumor cells. *Proc Natl Acad Sci USA* 1994;91:4318–22.
11. Weijtens MEM, Willemsen R, Valerio D, Stam K, Bolhuis R. Single chain Ig-γ gene-redirected human T lymphocytes produce cytokines, specifically lyse tumor cells, and recycle lytic capacity. *J Immunol* 1996;157:836–43.
12. Altenschmidt U, Kahl R, Moritz D, et al. Cytolysis of tumor cells expressing the Neu/erbB-2, erbB-3, and erbB-4 receptors by genetically targeted naive T lymphocytes. *Clin Cancer Res* 1996;2:1001–8.
13. Yang O, Tran A-C, Kalams S, et al. Lysis of HIV-I-infected cells and inhibition of viral replication by universal receptor T cells. *Proc Natl Acad Sci USA* 1997;94:11478–83.
14. Hombach A, Heuser C, Sircar R, et al. An anti-CD30 chimeric receptor that mediates CD3-independent T-cell activation against Hodgkin's lymphoma cells in the presence of soluble CD30. *Cancer Res* 1998;58:1116–19.
15. Hwu P, Yang J, Cowherd R, et al. In vivo antitumor activity of T cells redirected with chimeric antibody/T-cell receptor genes. *Cancer Res* 1995;55:3369–73.

16. Restifo NP, Kawakami Y, Marincola F, et al. Molecular mechanisms used by tumors to escape immune recognition: immunogene therapy and the cell biology of the major histocompatibility complex class I. *J Immunother* 1993;14:182–90.
17. Nolan KF, Yun CO, Akamatsu Y, et al. Bypassing immunization: optimized design of designer T cells against carcinoembryonic antigen (CEA)-expressing tumors, and lack of suppression by soluble CEA. *Clin Cancer Res* 1999;5:3928–41.
18. Akamatsu Y, Nolan KF, Murphy JC, et al. A single chain immunotoxin from a humanized antibody against carcinoembryonic antigen that suppresses growth of colorectal carcinoma cells. *Clin Cancer Res* 1998;4:2825–32.
19. Sharkey RM, Juweid M, Shevitz J, et al. Evaluation of a complimentarity-determining region-grafted (humanized) anti-carcinoembryonic antigen monoclonal antibody in preclinical and clinical studies. *Cancer Res* 1995;55(Suppl):5935–45.
20. Coligan JE, Kruisbeek AM, Margulies DH, et al., eds. *Current Protocols in Immunology.* St. Louis: John Wiley and Sons, 1998.
21. Niles RM, Wilhelm SA, Steele GD, et al. Isolation and characterization of an undifferentiated human colon cancer cell line (MIP-101). *Cancer Invest* 1987;5:545–52.
22. Thomas P, Gangopadhyay A, Steele G, et al. The effect of transfection of the CEA gene in the metastatic behavior of the human colorectal cancer cell line MIP-101. *Cancer Lett* 1995;92:59–66.
23. Ballas ZK, Rasmussen W, Van Otegham JK. Lymphokine activated killer cells. II. delineation of distinct murine LAK-precursor subpopulations. *J Immunol* 1987;138:1647–52.
24. Sawada H, Abo T, Sugawara S, Kumagai K. Prerequisite for the induction of lymphokine activated killer cells from T lymphocytes. *J Immunol* 1988;140:3668–73.
25. Yamamoto RS, Coss J, Vayuvegula B, et al. Generation of stimulated activated T killer (T-LAK) cells from the peripheral blood of normal donors and adult patients with recurrent glioblastoma. *J Immunol Methods* 1991;137:225–35.
26. Toribio ML, De Landazuri MO, Lopez-Botet M. Induction of natural killer-like cytotoxicity in cultured human thymocytes. *Eur J Immunol* 1983;13:964.
27. Havele C, Bleackley RC, Paetkau V. Conversion of specific to non-specific cytotoxic T lymphocytes. *J Immunol* 1986;137:1448.
28. Burns GF, Triglia T, Werkmeister JA. In vitro generation of human activated lymphocyte killer cells: separate precursors and modes of generation of NK-like cells and anomolous killer cells. *J Immunol* 1984;133:1656.
29. Damle NK, Doyle LV, Bradley EC. Interleukin 2-activated human killer cells are derived from phenotypically heterogeneous precursors. *J Immunol* 1986;137:2814.
30. Strand S, Hofmann WJ, Hug H, et al. Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells—a mechanism of immune evasion? *Nat Med* 1996;2:1361–6.
31. Hahne M, Rimoldi D, Schroter M, et al. Melanoma cell expression of Fas(Apo-1/CD95) ligand: implications for tumor immune escape. *Science* 1996;274:1363–6.
32. Torre-Amione G, Beauchamp RD, Koeppen H, et al. A highly immunogenic tumor transfected with a murine TGF cDNA escapes immune surveillance. *Proc Natl Acad Sci USA* 1990;87:1486–90.

COUPLING CD28 COSTIMULATION TO IgTCR MOLECULES: DYNAMICS OF T CELL PROLIFERATION AND DEATH

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Abstract:

IgTCR molecules are potentially potent immune response modifiers because they endow the T cell with the ability to bypass tolerance. Tolerance to self antigens has been one of the major barriers to developing effective adoptive immunotherapies for cancer. In vitro studies in a number of labs have shown that cross linking IgTCR molecules with the target antigen leads to cytolytic activity, cytokine release, and T cell proliferation in model systems. However, many of these studies have utilized established T cell lines rather than normal T cells and/or indirect assays of cytotoxicity, proliferation and cytokine release. We have sought to establish the validity of these model systems in the context of developing more effective adoptive immunotherapies with normal human T cells. The present study examines the activation of T cell proliferation following IgTCR cross linking. The results show that, in addition to IgTCR signals, CD28 costimulation is required to induce expansions of normal PBMC-derived T cells. IgTCR signals alone can induce transient cell division, but they do not induce the prolonged polyclonal expansions that are characteristic of native immune responses. Very strong IgTCR signals could circumvent the CD28 requirement, but only at levels that are unlikely to be physiologically relevant. CD28 costimulation also suppressed the deletion of tumor-reactive subclones by activation induced cell death (AICD). These studies confirm the importance of CD28 costimulation to the proliferation of IgTCR-modified human T cells, a key feature to an effective, reconstructed anti-tumor response.

Key words:
Immunoglobulin T cell receptor
CD28
Activation
Proliferation
Clonal selection

INTRODUCTION

The hallmarks of an effective cellular immune response are: 1) cytolytic activity (CTLs), 2) T cell proliferation, and 3) cytokine release. Each of these T cell functions plays an important role in determining the overall success of the response. CTL functions are responsible for physically eliminating pathogens or infected cells. Cytokines have a wide range of functions, but generally serve to promote the growth or differentiation of different immune cell subsets. Proliferation, is crucial to the expansion of responsive T cell clones in numbers adequate to eliminate an infectious agent.

Immunoglobulin T cell receptors (IgTCR) are chimeric molecules that consist of the antigen binding portion of an antibody fused to one of the chains of the T cell receptor. The binding properties of IgTCR molecules provides several advantages for adoptive immunotherapy for cancer. First, the large number of possible antibody combinations allows T cell specificity to be assigned to virtually any antigen. Most tumor antigens are self proteins and T cells capable of recognizing these self antigens have either been deleted or have become tolerant. Thus, the selection of the appropriate antibody specificity allows IgTCR molecules to bypass tolerance. A second major advantage is that IgTCR-modified T cells have greater autonomy than normal T cells. This is because IgTCR-modified cells are activated directly by the immunologic target

rather than through a complex process of antigen presentation by accessory cells. Lastly, antigen binding is much stronger for IgTCR molecules than for the normal TCR, since antibody affinities are several orders of magnitude higher than that of a typical TCR molecule. Higher binding affinity may allow IgTCR molecules to function independently of the adhesion/costimulatory molecules usually required to facilitate normal TCR binding.

A large number of studies have examined the ability of IgTCR molecules to activate T cells (1-16). Direct cross-linking of the chimeric receptor by the target antigen leads to potent antigen-specific CTL activity (3-13, 15), and the release of cytokines such as IL2 (2, 3, 5, 7, 13, 16), γINF (7), GM-CSF (6, 10), and TNFα (1, 9). In addition, two studies (7, 8) have reported that IgTCR cross linking induces T cell proliferation. The ability of these molecules to singlehandedly induce all major T cell functions suggests that IgTCR activation may be less dependent upon the participation of costimulatory or adhesion molecules such as CD28, LFA-1, and CD2.

When normal T cells encounter antigen they respond in one of several ways. These reactions can include complete activation, partial activation, anergy, or the induction of apoptosis through a process termed activation induced cell death (AICD). Which reaction occurs is determined by the types of signals the cell receives. In addition to TCR ligation (signal 1), T cells usually require one or

more costimulatory signals to become fully activated. The most well defined of these is delivered through the CD28 receptor (signal 2) (reviewed in 17, 18). When T cells do not receive CD28 costimulation, a state of unresponsiveness or anergy is usually induced (17, 18). However, increasing the strength of signal 1 can lead to a reduced dependence on CD28 costimulation with enhanced proliferation and IL2 production in the absence of CD28 costimulation (19-23).

One implication of the aforementioned studies is that the higher binding affinity of IgTCR molecules may allow them to bind tightly to antigen and transmit very strong signal 1. We examined the hypothesis that such strong IgTCR signals might reduce the dependence on CD28 and be sufficient to independently induce proliferation in normal T cells. In the present study, proliferation was examined after stimulation with different combinations of IgTCR and CD28 activation signals. The results confirm the central role of CD28 (signal 2) for sustained ex vivo proliferation that can only be partly superceded by strong signal 1. These studies add to our understanding of the key immune elements necessary to designing effective anti-tumor T cell gene therapies in humans.

MATERIALS AND METHODS

Retroviral Vector and Vector Producer Cells

The construction of anti-CEA IgTCR genes has been previously described (15, 16). The current studies were conducted with an IgTCR gene containing sequences encoding the heavy and light chain variable regions (joined by a flexible linker) from the humanized MN14 antibody fused to sequences encoding the ζ-chain of the human T cell receptor (16). The antibody and ζ-chain sequences are separated by the hinge region of CD8α. The anti-CEA sFvζ construct was inserted into the MFG retroviral vector backbone as previously described (15). Retroviral vector supernate was produced using the PG13 producer cell line (15).

The pLNSX-hB7.1 vector (kindly provided by Dr. Lieping Chen, Mayo Clinic, Rochester, MN) contains a cDNA copy of the human B7-1 gene inserted into the LNSX vector backbone. LNSX-hB7.1 vector producer cells were constructed by transfecting the vector into GPE86 ecotropic helper cells and using the transient viral supernate to infect the PA317 packaging cell line. PA317 cells were then selected with G418 for 10 days after which viral supernates were harvested and used to transduce the MIP-CEA and MIP-101 tumor cell lines.

Lymphocyte transduction and Culture

Peripheral blood mononuclear cells (PBMC) from normal blood were cultured and transduced as previously described (15). Following transduction, T cells were expanded in growth media (GM, AIMV supplemented with 10% Fetal calf serum, and 300 U/mL recombinant IL2) until the cells reached plateau phase and rapid cell proliferation caused by the initial OKT3-induced activation had ceased. T cells were then used in proliferation assays. Individual preparations of T cell cultures were slightly variable, but generally consisted of ≈40-50% IgTCR-positive cells, of which approximately 60% were CD4+ and 40% CD8+ (data not shown).

Tumor Cell Lines and Transductions

MIP-101 is a poorly differentiated human colorectal cancer cell line that does not express CEA (24). The MIP-CEA cell line was derived by transfecting MIP-101 with a full length cDNA encoding the human CEA gene (25). Tumor cells were cultured as previously described (15). Tumor cell lines expressing the human B7.1 gene (MIP-101.B7 and MIP-CEA.B7) were constructed by infecting parental MIP-101 and MIP-CEA cells respectively with a B7.1-containing retroviral vector (LNSX-hB7.1). Expression of the CEA and B7.1 proteins in tumor cells was confirmed by staining the cells with PE-conjugated hMN14 and FITC-conjugated anti-human B7.1 antibody, and then analyzing the cells by FACS.

Antibodies and Flow cytometry

Humanized MN14 (hMN14) antibody and its anti-idiotype antibody, WI2, were obtained from Immunomedics (Morris Plains, New Jersey). WI2 is designated as anti-IgTCR in the text. DAB9.3 is a mouse antibody against the human CD28 receptor (kindly provided by Dr. Carl June, Univ of Pennsylvania, Philadelphia, Pennsylvania). DAB9.3 was used as a binding analog of the human B7 antigens in experiments employing plate bound antigens. OKT3 (Ortho Biotech Inc. Raritan, NJ.) is a mouse antibody directed against the normal human T cell receptor, and was used as a binding analog for antigen-MHC complexes in experiments employing plate bound antigens. UPC-10 (Sigma, St. Louis, Missouri) is a mouse antibody with binding specificity for β-2-6 linked fructosan and was used as a negative control staining antibody in experiments utilizing mouse antibodies. HAT (Hoffmann-La Roche Inc., Nutley, New Jersey) is a humanized anti-Tac antibody and was used as a negative control staining antibody for experiments utilizing hMN14. Anti-human B7.1 antibody was obtained from Ancell Corp. (Bayport, Minnesota). Fluorescein isothiocyanate (FITC) and P-phycoerythrin (PE) labeled antibodies against different human T cell antigens (CD4, CD8) and against mouse and human Fc were obtained from Caltag Laboratories (Burlingame, California). All antibody staining reactions were performed using standard methods (26). Fluorescence intensity was measured using a Coulter EPICS Profile II flow cytometer.

T Cell Proliferation Assays

Proliferation assays using plate-bound antigens. 96 well tissue culture plates were coated with antibodies by incubation overnight in 0.1 M sodium bicarbonate buffer (pH 7.0), followed by washing with fresh media. Cells were added to replicate wells to allow for an undisturbed well to be harvested on each day for which cell counts were to be performed. At specified times, T cells were harvested from wells by vigorous pipetting to remove bound cells from the plate surface and to create a single cell suspension. Viable cells were quantified by trypan blue exclusion. When possible, a minimum of 100 cells were counted for each test sample. Individual wells were counted from 2 to 9 times depending on the cell density. For wells that contained a high density of cells, 2 duplicate counts were performed. In cases where extensive cell death, or a lack of proliferation made it difficult to obtain >100 cells for counting, a minimum of 9 counts were done. These cell counts were then averaged to obtain the total number of viable cells for each time point and treatment group in a given experiment. Cell counts from repeated independent experiments were then averaged for each time point/treatment group and the number of viable cells plotted as a function of time.

Proliferation assays using tumor bound antigen. Tumor cell lines were seeded into Falcon 1013 150mm tissue culture plates and incubated overnight at 37°C to allow time for the cells to adhere and begin proliferating. 24 hours later

IgTCR-modified T cells were added to the culture plates, and the plates were returned to incubation at 37°C. During tumor cell/T cell co-incubation periods, GM was used for culture media in order to maintain the activational state of the T cells. Both MIP-101 and MIP-CEA proliferate normally in this media. T cells and tumor cells were counted every few days by trypsinizing the plates to create single cell suspensions, mixing an aliquot of the suspension with trypan blue, and counting the cells as described above. Both MIP-101 and MIP-CEA cells are 2-3 fold larger than human T cells and can be readily distinguished from T cells using light microscopy.

RESULTS

The central objective of these studies was to determine which activation signals are required to induce proliferation in normal human T cells. The premise that IgTCR signals alone are sufficient for proliferation is supported by the numerous in vitro studies showing IgTCR cross linking activates all major T cell responses (1-16). However, these previous reports of IgTCR-induced proliferation are based on increased ^{3}H-thymidine incorporation (7, 8) in IgTCR stimulated cells. ^{3}H-incorporation assays measure DNA replication transiently, and even high levels of ^{3}H-incorporation may not accurately reflect the logarithmic expansion of T cells that occurs during a native immune response. Further, when measured early in the response, even anergizing signals that lead to abortive proliferative responses, can induce levels of ^{3}H-incorporation that are similar to those obtained with non-anergizing signals (27). The present studies have utilized direct cell counting methods, rather than ^{3}H-incorporation to measure proliferation. Our overall goal in these studies was to evaluate proliferation from an immunologically relevant standpoint. This requires the ability to distinguish between true logarithmic expansions of cells and abortive proliferative responses that may involve DNA replication, but which ultimately end in AICD. Direct cell counting methods are more effective than ^{3}H-incorporation assays for accomplishing this.

As a model, we have used an IgTCR molecule directed against the carcinoembryonic antigen (CEA) (15, 16). To distinguish between transient replication and true logarithmic expansions, direct cell counting methods over long periods of time were utilized. To analyze proliferation under physiologically relevant conditions, PBMC-derived T cells were used rather than transformed T cell lines, and antigenic stimuli were displayed on the surface of tumor cells. T cells used in the present studies were cultured for ≈20-30 days prior to the start of any experiments so that the cultures would reach plateau phase and cease the rapid proliferation caused by the initial anti-CD3 activation. At this stage, cultures of PBMC-derived T cells are generally in decline and consist of two populations of cells: (1) dead and dying cells, and (2) viable, resting cells. T cells that remain viable in these plateau cultures have generally returned to a resting state (single cell suspension of small rounded cells), but are still capable of reactivating when stimulated with antigen (see ref 15).

IgTCR expression in normal peripheral blood T lymphocytes

IgTCR expression in cultures of primary human T lymphocytes was measured by flow cytometry. Following two rounds of infection, approximately 40-50% of the T cells express the anti-CEA IgTCR gene (Fig.1). The ratio of CD4+ and CD8+ cells in different preparations of T cells was generally ≈60% CD4 and 40% CD8 (data not shown). Expression of the anti-CEA IgTCR gene was

stable in long term cultures of primary human T cells, remaining virtually unchanged over a period of >2 months (data not shown). All T cell experiments described in this report were performed using unselected populations of T cells with proportions of IgTCR-negative and IgTCR-positive cells similar to the population shown in Fig.1.

IgTCR-mediated stimulation of T cells results in cell death rather than proliferation

Initial experiments focused on determining whether or not stimulating T cells with only IgTCR signals would induce T cell proliferation. Gene-modified T cells were incubated with either immobilized anti-IgTCR antibody, or with MIP-CEA tumor cells (Fig.2). In both cases, ligation of the IgTCR receptor by the target antigen led to increased T cell death rather than proliferation. In contrast, the same T cells that were cultured in IL2-containing media alone (not exposed to antigen) continued to proliferate slowly. Although T cells co-incubated with tumor cells progressively die, they nonetheless retain potent anti-CEA CTL activity for a number of days (see ref. 15 and below)

Stimulation of T cells through both the IgTCR and CD28 receptors induces rapid and sustained proliferation.

To determine if co-stimulating T cells through both the IgTCR and CD28

receptors would induce T cell proliferation, tissue culture plates were coated with either anti-CD28 antibody, anti-IgTCR antibody, or both antibodies together. T cells were bound to the plates and cultured in media containing 300 U/mL of recombinant IL2. The number of viable T cells in each treatment was then measured relative to control cultures that were not stimulated (Fig.3). Data points are plotted as percent of control to normalize the cell counts for the slightly different rates of background proliferation in different replicate experiments. T cells stimulated with anti-IgTCR antibody alone (signal 1) proliferated only slightly, while cells stimulated with anti-CD28 alone (signal 2) showed no proliferative response. In contrast, T cells stimulated with both anti-IgTCR and anti-CD28 antibodies (signals 1 and 2) began to rapidly proliferate after ≈5 days and continued proliferating until the experiment was halted on day 18. Analogous control experiments on the same cells, but using anti-CD3 antibody, with and without anti-CD28, gave virtually identical results. This demonstrates that the IgTCR and normal TCR signaling pathways function similarly and are both influenced by the presence, or absence of CD28 costimulation. It was possible to induce prolonged proliferation with signal 1 alone but only when plates were coated with antibody concentrations significantly higher than those used in Figure 3 (see below). This is in agreement with previous studies on the normal TCR that have shown that signal 1 alone can induce T cell proliferation if the signal strength is strong enough (22, 27).

Previous studies have indicated that immobilization of antigens on solid surfaces such as plastic culture plates can artificially increase signal strength (21-23). To measure proliferation under more physiologically relevant conditions. a second set of experiments was performed in which ligands for signal 1 and signal 2 receptors were displayed on tumor cells. T cells were co-incubated with four different tumor cell lines which displayed surface ligands capable of crosslinking either the IgTCR molecule and/or the CD28 receptor. To generate the appropriate tumor bound ligands, CEA-negative (MIP-101) and CEA-positive (MIP-CEA) colon carcinoma cell lines (24, 25) were transduced with a retroviral vector carrying the human B7-1 gene. After selection, appropriate surface expression of the CEA and B7-1 proteins was confirmed by FACS (Fig.4).

The growth curves of T cells co-incubated with tumor cells expressing CEA alone (signal 1), or CEA and B7.1 (signal 1 and signal 2) are shown in Fig.5A. T cells stimulated with signals 1 and 2 slowly proliferated over the course of the experiment. In contrast, T cells stimulated by signal 1 alone slowly declined in number. T cells incubated with CEA-negative, MIP-101 (no signal) or MIP-101-B7 tumor cells (signal 2 alone) both remained relatively unchanged until the plates became overgrown with tumor and were discarded on day 8 (data not shown).

Activation through both the IgTCR and CD28 receptors increases anti tumor efficacy

The differential response of T cells to tumors expressing CEA-alone or CEA and B7 was also reflected in the growth or death of the respective tumor lines themselves. CEA-expressing tumor cells are rapidly lysed by anti-CEA IgTCR-modified T cells (15). To maintain antigenic stimulation, these co-cultures were periodically fed with fresh MIP-CEA or MIP-CEA-B7 tumor cells. T cells stimulated with signal 1 alone were able to lyse tumor cells for only 7-10 days before the continuous addition of tumor cells (MIP-CEA) eventually overwhelmed the lytic capacity of the T cells (Fig. 5B). After this, the tumor cells rapidly proliferate and eventually overgrow the cultures. In contrast, T cells stimulated with signal 1 and 2 (MIP-CEA-B7) were more effective at killing tumor cells (Fig. 5C). In these co-cultures, MIP-CEA-B7 tumor cells were added on 7 different occasions over the course of the experiment without the outgrowth of tumor. More tumor cell killing in these cultures was mainly due to T cell proliferation and the resulting maintenance of more favorable effector-to-target cell ratios. Counts of viable tumor cells displaying no ligand (MIP-101), and only signal 2 ligand (MIP-101-B7) showed that both tumor cells lines proliferate rapidly, such that by day 8, the cultures were discarded due to the overgrowth of tumor cells (data not shown).

Signal 1 and 2 act in concert to reduce the total amount of signal required

to activate proliferation

Under some circumstances, T cells can be activated to proliferate by signal 1 alone (21-23). Yet it is also clear that signal 2 plays a major role in controlling T cell growth (see Fig.3). To define the relative roles of signal 1 and 2, proliferation assays were performed in which the strength of signal 1 was progressively increased either in the presence or absence of a constant level of signal 2 (Fig.6). This was accomplished by assaying T cell growth in tissue culture plates coated with increasing concentrations of anti-IgTCR antibody alone (signal 1 only), or with anti-IgTCR and anti-CD28 antibodies together (signal 1 and 2). In T cells stimulated with signal 1 alone, no T cell proliferation was observed until the anti-IgTCR antibody concentration reached very high levels (5-20 ug/mL). When T cells were stimulated with signal 1 and 2, proliferation was induced at ≈500-fold lower levels of signal 1 (0.01-0.05 ug/mL). This demonstrates that signal 2 serves to lower the amount of signal 1 required to trigger proliferation.

In both instances, proliferation was not induced until a specific level of signal 1 strength was reached (Proliferation threshold). One interesting aspect of this analyses is that the range of antigen concentration between no growth, and the maximum rate of growth was fairly broad (≈100-fold increase in antigen concentration from minimum to maximum proliferative rates). Further, in both cases there appeared to be intermediate levels of proliferation within these broad

"proliferation-induction zones". Thus, signal strength appears to regulate both the intensity of the response, as well as its induction. The intensity of the response is finite however, since once the maximum rate of proliferation was reached, the cells proliferate at about the same rate regardless of whether or not signal strength was further increased. The maximum rate of proliferation shown in Fig.6 is about a 40% increase in cell numbers per day. Even in the presence of adequate levels of signal 1 and signal 2, there is no net increase in cell number during the first 4-5 days (see Fig.3). The values shown in Fig.6 represent average cell growth over a 10 day period, including several days of no net growth. By day 10, maximally stimulated T cells were generally doubling about once every 18 hours (data not shown). Thus, once a proliferative burst is underway, T cell growth can be explosive.

These results demonstrate that T cell proliferation is triggered by signal 1. This can be accomplished by very high levels of signal 1 alone, or at much lower levels when both signal 1 and signal 2 are combined. Virtually identical results were obtained in control experiments where T cells were stimulated through the normal TCR, by binding to anti-CD3 antibody alone or in combination with anti-CD28 antibody (data not shown). This indicates that both the normal and chimeric signaling pathways function similarly.

While a specific level of signal 1 and signal 2 was required to activate

proliferation, other indicators of T cell activation could be induced a much lower levels. In parallel to proliferation, morphological correlates of activation were also examined. When resting T cells are activated they convert from a single cell suspension of small, round cells to aggregates of large, metabolically active blast cells. Activation-induced changes in morphology were quantified by analyzing forward-scatter/log side-scatter (FS-LSS) flow cytometric plots of cells stimulated with different signals.

Fig. 7A shows the FS-LSS plot for control cells which were not stimulated with either signal 1 or signal 2. T cells used for these assays (Figures 6, 7 and 8) were obtained from plateau phase cultures which contain a large proportion of dead and dying cells (arrow in Fig. 7A). T cells that remain viable in these plateau cultures have a small rounded shape (circled population in Fig. 7A). When these same cells were stimulated with either signal 1 alone (Fig.7B), or with signal 1 and signal 2, (Fig.7C, non-proliferating cells, Fig.7D proliferating cells) they uniformly converted to a blast cell morphology (circled populations) irrespective of whether or not the cells proliferated. By our evaluation, signal 2 had no impact on morphological change. Thus, different T cell functions are activated at different levels of signal strength. Some functions (eg. morphologic activation) are induced by weak or incomplete signals, while other functions (eg. proliferation) require stronger or more complete signals. Similar results were obtained in control experiments where T cells were stimulated through the

normal TCR alone (by anti-CD3 antibody) with and without CD28 costimulation (data not shown), demonstrating that the IgTCR activation pathway mirrors events that occur in the normal activation pathway.

Selective expansion of IgTCR-positive cells with two signals

The foregoing experiments suggest an antigen-specific deletion by AICD when T cells receive signal 1 only (Fig.2 and 5), or an expansion of reactive clones in the presence of costimulation (Figs. 3, 5 and 6). As a test of these principles, we examined changes in the relative proportion of IgTCR+ cells in the absence or presence of costimulation. The proportion of IgTCR-positive and -negative T cells at the start of these experiments was ≈50:50 (Fig.8A). T cells stimulated through the IgTCR receptor alone underwent a progressive shift to a population of cells that were predominantly IgTCR-negative (Fig 8B). In contrast, T cells stimulated through both the IgTCR and CD28 receptors shifted to a population that was 100% IgTCR-positive (Fig. 8C). As in the experiments above, control experiments in which T cells were stimulated through either the normal TCR alone (Fig.8D), or through the normal TCR and CD28 receptors (Fig.8E). However, in the present experiments cells stimulated through the normal TCR reacted differently than those stimulated through the chimeric receptor. T cells stimulated through the normal TCR remained virtually unchanged from the start of the experiments at an ≈50:50 ratio of IgTCR-positive and -negative cells. With the normal TCR, proliferation, or AICD affects IgTCR-positive and -

negative populations equally and hence, does not change the proportion of positive and negative cells.

DISCUSSION

As one of the three major T cell responses, antigen-induced proliferation is a critical component of cellular immunity. Antigen specific T cell proliferation is particularly important for adoptive immunotherapy since a number of studies have shown that ex vivo modified T cells do not circulate well after infusion (28-31). This poor circulation may limit the distribution of modified cells into tumor tissues. In the absence of a proliferative response, the few cells that actually infiltrate tumor tissues may be insufficient to accomplish significant tumor reduction.

Inducing T cell proliferation with IgTCR molecules requires CD28 co-stimulation.

Previous studies (7, 8) have reported that IgTCR cross linking alone is sufficient to activate antigen specific proliferation. The present study indicates that a productive expansion of IgTCR-modified cells does not generally occur unless the cells also receive CD28 costimulation. T cell proliferation induced by IgTCR+CD28 activation represents a true expansion in cell number, rather than transient replication that can occur with abortive responses that end in T cell death. Proliferation could be induced by signal 1 alone (both anti-IgTCR and anti-CD3), but only when high concentrations of cross linking antibody was immobilized on a solid surface, similar to results obtained by others (21-23).

However, these high signal strengths are unlikely to be physiologically relevant. This conclusion is supported by the lack of proliferation when T cells were incubated with MIP-CEA tumor cells. MIP-CEA tumor cells express very high levels of cell surface CEA (see ref. 15), yet were unable to induce proliferation of T cells unless the tumor cells also displayed the B7-1 protein (Fig. 5A).

Role of costimulation is to reduce the level of signal required to allow proliferation.

The two signal model holds that costimulation reduces the signaling threshold required to activate T cell proliferation (see refs.17, 18). The present studies confirm that IgTCR-induced proliferation is similarly constrained. However, while signal 1 and 2 are required for T cell proliferation to occur, costimulation does not lead to fully autonomous cell division. T cell proliferation was still dependent upon the addition of exogenous IL2, and rapidly ceased upon IL2 withdrawal (data not shown). This is consistent with recent reports indicating that, in addition to TCR and CD28, LFA-1 cross linking is also required to induce the release of effective quantities of IL2 (32, 33). Although costimulation does not fulfill all signal requirements, it does appear to be essential for proliferation at physiologic levels of antigen, since its absence could not be overridden by the presence of exogenous IL2.

Using an IgTCR molecule against prostate specific membrane antigen, Gong et.

al., (34) have reported that IL2 release is induced by coupling CD28 costimulation to IgTCR signals. However, the amount of IL2 produced was only about 3000 pg/ml/10^6 cells/24 hours (by ELISA). IL2 release in this study also began to decline after 48 hours. 3000 pg/ml of IL2 is equal to about 45 IU/ml of IL2, which is not sufficient, in our experience, to maintain T cell proliferation (unpublished data). Our studies uses different criteria to define IL2 release. Specifically, we use T cell proliferation in the absence of exogenous IL2 as a measure of the biologic consequence of endogenous IL2 production. This is a more stringent assay readout than ELIZA because the T cells must synthesize and release enough endogenous IL2 to maintain autonomous growth. It is likely that CD28 costimulation does induce the release of some endogenous IL2, particularly since other studies have shown that CD28 costimulation plays a role in regulating the transcription and stability of IL2 mRNA (35-37). However, our results indicate that the amount of IL2 production induced by IgTCR+CD28 crosslinking is insufficient for maintaining T cell proliferation.

Incorporating CD28 co-stimulation leads to an increase in anti-tumor efficacy

Incorporating CD28 signaling into the IgTCR activation pathway increases the overall ability of IgTCR-modified T cells to kill tumor. The co-culture experiments shown in Fig. 5 represent an in vitro model of therapy in which a

defined "dose" of T cells are added to tumor cells with and without costimulation. T cells stimulated by signal 1 and 2 lysed a greater total number of tumor cells, and maintained their potency for a much longer period of time, whereas T cells stimulated by signal 1 alone were overwhelmed by the rapidly dividing tumor cells. Fig. 5 also shows that many more tumor cells were added to the MIP-CEA-B7 co-cultures than to the MIP-CEA cultures. When the difference in the number of tumor cells added, and the growth potential of those cells is taken into account, there is a ≈200-fold difference in anti-tumor activity between T cells stimulated with signal 1 and 2, and those stimulated with signal 1 alone. The similar rates of tumor lysis, at fixed E:T ratios, in both types of co-culture have led us to conclude that the increased anti-tumor efficacy seen with signal 1 and 2 is due to improved growth and survival of the T cells, rather than increased activity of CTLs on a per cell basis. These results highlight the importance of T cell proliferation to an effective anti-tumor response.

Individual T cell effector functions have individual signaling requirements. While T cell proliferation required a minimum level of signal 1, the presence of signal 2, and exogenous IL2, other T cell functions could be activated without this collaboration. When morphologic criteria are used to define T cell activation, signal 1 is just as effective as signal 1 and signal 2 together, and all cells become morphologically activated regardless of whether or not they proliferate. This indicates that T cell activation proceeds via a stepwise

progression in which signal 1 is sufficient to induce some functions while costimulation is required to turn on higher order responses such as proliferation. This makes sense in that TCR recognition (signal 1) must be the first event in the immune response and its stimulation prepares the cell (e.g. up-regulation of IL2R, CD28, LFA-1, metabolic rate, etc.) to participate in antigen driven proliferation.

Specific signals control T cell entry into either an AICD pathway, or a selective expansion of reactive clones

Analysis of dynamic changes in the proportion of IgTCR+ cells showed that stimulation with signal 1 alone leads to a progressive and selective deletion of antigen reactive clones (Fig. 8B, IgTCR-only). These cultures were not proliferating and the conversion of the population to predominately IgTCR-negative cells resulted from the selective death of IgTCR+ cells. In contrast, T cells stimulated with signal 1 and 2 progressively shifted from a 50:50 mixture of IgTCR-negative and IgTCR-positive cells to a population that was 100% IgTCR+ (Fig. 8B, IgTCR+CD28) through the selective outgrowth of T cells that expressed higher levels of the receptor. Subclones that express quantitatively higher levels of receptor can generate stronger signals than subclones that express low levels of receptor simply because there are more receptors that can be crosslinked. This stronger signaling potentially provides high expressing cells with a selective advantage. When costimulation is present,

this translates into a growth advantage for high expressors. When costimulation is absent, AICD results in the deletion of high expressors.

These dynamic changes are similar to events that take place during normal immune responses. Previous studies have shown that the repertoire of antigen-responsive subclones will evolve during in vivo T cell expansions. This evolution involves dynamic changes in the proportions of different subclones such that the response becomes focused on specific epitopes (38-40). In some responses, this repertoire focusing is caused by the selective expansion of subclones whose TCR$\alpha\beta$ chains have higher binding affinities (41). In contrast, the evolution of high expressing IgTCR subclones in Fig.8 does not arise because IgTCR molecules vary in their affinity for antigen. All IgTCR subclones express the same chimeric receptor with the same receptor affinity. Rather, IgTCR subclones differ in the total number of receptors expressed per cell and high levels of receptor on specific subclones leads to stronger binding, higher signal strength, and more rapid proliferation in those clones. By this mechanism, high expressing subclones evolve to become the predominant subtypes. Thus, the end result of increased TCR cross linking is analogous between the two systems, despite the qualitative differences between them.

In summary, the present study demonstrates that CD28 costimulation is required to induce proliferation of IgTCR-stimulated human T cells. Previous reports

that show ^{3}H-thymidine incorporation (7, 8) after IgTCR cross linking most likely measured transient DNA replication that can occur during abortive responses (27). Proliferative responses that occur with IgTCR and CD28 costimulation are prolonged expansions that more closely resemble what occurs during a productive native immune response. The present study also shows that IgTCR-induced activation proceeds in a stepwise fashion, in which weaker signals will induce morphological activation, but not proliferation. We previously showed that cytolytic functions are fully induced by IgTCR signals alone and do not require either CD28 or exogenous IL2 (15). Thus, the first step of T cell activation appears to involve a group of responses that include morphologic, metabolic, and cytolytic functions. Productive proliferation, on the other hand, appears to be one of the final steps of activation and requires, not only signal 1, signal 2, and sufficient quantities of IL2, but also a level of signal 1 that is above a specific threshold. This threshold also appears to be the point at which the T cell commits to either an AICD pathway (absence of signal 2), or to a pathway of clonal expansion and affinity selection (with signal 2). Lastly, the integration of CD28 costimulation into the IgTCR-activation pathway may increase anti-tumor responses in clinical applications of IgTCR-based immunotherapies. We are currently testing the ability of IgTCR/IgCD28 dual chimera vectors to direct antigen specific cytolysis and proliferation. Because IL2 is not released in this setting, a third level of activation may be required for a more complete self-sustaining anti-tumor response that does not

require the addition of exogenous IL2.

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REFERENCES

1. Kuwana, Y., Asakura, Y., Utsunomiya, N., Nakanishi, M., Arata, Y., Itoh, S., Nagase, F., and Kurosawa, Y. Expression of chimeric receptor composed of immunoglobulin-derived V regions and T cell receptor derived C regions. *Biochem. Biophys. Res. Commun. 1987*:149:960-968.

2. Gross, G., Waks, T., and Eshhar, Z. Expression of immunoglobulin-T-cells receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc. Natl. Acad. Sci. USA*. 1989:86:10024-10028.

3. Goverman, J., Gomez, S., Segesman, K., Hunkapiller, T., Laug, W., and Hood, L. Chimeric immunoglobulin-T cell receptor proteins form functional receptors: Implications for T cell receptor complex formation and activation. *Cell*. 1990:60:929-938.

4. Romeo, C., and Seed, B. Cellular immunity to HIV activated by CD4 fused to T cell or Fc receptor polypeptides. *Cell*. 1991:64:1037-1046.

5. Eshhar, Z., Waks, T., Gross, G., and Schindler, D. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the γ or ζ subunits of the immunoglobulin and T-cell receptors. *Proc. Natl. Acad. Sci. USA*. 1993:90:720-724.

6. Hwu, P., Shafer, G., Treisman, J., Schindler, D., Gross, G., Cowherd, R., Rosenberg, S., and Eshhar, Z. Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor γ chain. *J. Exper. Med.* 1993:178:361-366.

7. Roberts, M., Qin, L., Zhang, D., Smith, D., Tran, A-C., Dull, T., Groopman, J., Capon, D., Byrn, R., and Finer, M. Targeting of human immunodeficiency virus-infected cells by CD8+ T lymphocytes armed with universal T-cell receptors. *Blood.* 1994:84:2878-2889.

8. Becker, M.L.B., Near, R., Mudgett-Hunter, M., Margolies, M.N., Kubo, R., Kaye, J. and Hedrick S.M. Expression of a hybrid immunoglobulin-T cell receptor protein in transgenic mice. *Cell* 1989:58:911-921.

9. Moritz, D., Wells, W., Mattern, J., and Groner, B. Cytotoxic T lymphocytes with a grafted recognition specificity for ERBB2-expressing tumor cells. *Proc. Natl. Acad. Sci. USA.* 1994:91:4318-4322.

10. Weijtens, M.E.M., Willemsen, R., Valerio, D., Stam, K., and Bolhuis, R. Single chain Ig/γ gene-redirected human T lymphocytes produce cytokines, specifically lyse tumor cells, and recycle lytic capacity. *J. Immunol.* 1996:157:836-843.

11. Altenschmidt, U., Kahl, R., Moritz, D., Schnierle, S., Gerstmayer, B., Wels, W., and Groner, B. Cytolysis of tumor cells expressing the Neu/erbB-2, erbB-3, and

erbB-4 receptors by genetically targeted naive T lymphocytes. *Clin. Can. Res.* 1996:2:1001-1008.

12. Yang, O., Tran, A-C., Kalams, S., Johnson, R., Roberts, M., and Walker, B. Lysis of HIV-I-infected cells and inhibition of viral replication by universal receptor T cells. *Proc. Natl. Acad. Sci. USA.* 1997:94:11478-11483.

13. Hombach, A., Heuser, C., Sircar, R., Tillmann, T., Diehl, V., Pohl, C., and Abken, H. An anti-CD30 chimeric receptor that mediates CD3-ζ-independent T-cell activation against Hodgkin's lymphoma cells in the presence of soluble CD30. *Can. Res.* 1998:58:1116-1119.

14. Hwu, P., Yang, J., Cowherd, R., Treisman, J., Shafer, G., Eshhar, Z., and Rosenberg, S. In vivo antitumor activity of T cells redirected with chimeric antibody/T-cell receptor genes. *Can. Res.* 1995:55:3369-3373.

15. Beecham, E.J., Ortiz-Pujols, S., and Junghans, R.P. Dynamics of tumor cell killing by human T lymphocytes armed with an anti-CEA chimeric immunoglobulin T cell receptor. J. Immunother. 1999:in press

16. Nolan, K.F., Yun, C.O., Akamatsue, Y., Murphy, J.C., Leung, S., Beecham, E.J., and Junghans, R.P. Bypassing immunization: Optimized design of "designer T cells" against carcinoembryonic antigen (CEA)-expressing tumors, and lack of suppression by soluble CEA. *Clin. Can. Res* 1999: submitted.

17. Sperling, A.I., and Bluestone, J.A. The complexities of T cell co-stimulation: CD28 and beyond. Immunol. rev. 1996:153:155-182.

18. Boussiotis, V.A., Freemena, G.J., Gribben, J.G., and Nadler, L.M. The role of B7-1/B7-2:CD28/CTLA-4 pahtways in the prevention of anergy, induction of productive immunity and down regulation of immune response. Immunol. Rev. 1996:153:5-26.

19. Green, J.M., Noel, P.J., Sperling, A.I., Walunus, T.L., Gray, G.S., Bluestone, J.A., and Thompson, C.B. Absence of B7-dependent responses in CD28-deficient mice. Immunity. 1994:1:501.

20. Lucas, P.J., Negishi, I., Nakayama, K., Fields, L.E., and Loh, D.Y. Naive Cd28-deficient T cells can initiate but not sustain an in vitro antigen-specific immune response. J. Immunol. 1995:154:5757

21. Goldstein J.S., Chen, T., Brunswick, M., Mostowsky, H., and Kozlowski, S. Purified MHC class I peptide complexes activate naive CD8+ T cells independently of the CD28/B7 and LFA-1/Icam-1 costimulatiory interactions. J. Immunol. 1998:160:3180-3187.

22. Luxembourg, A.T., Brunmark, A.B., Kong, Y., Jackson, P.A., Peterson, P.A., Sprent, J., and Cai, Z. Requirements for stimulating naive CD8 T cells via signal one alone. *J. Immunol.* 1998:161:5226.

23. Manickasingham, S.P., Anderton, S.M., Burkhart, C., and Wraith, D.C. Qualitative and Quantitative effects of CD28/B7-mediated costimulation on naive T cell in vitro. J. Immunol. 1998:161:3827-3835.

24. Niles, R.M., Wilhelm, S.A., Steele, G.D., Burke, B., Christensen, T., Dexter, D., O'Brien, M.J., Thomas, P., and Zamcheck, N. Isolation and characterization of an undifferentiated human colon cancer cell line (MIP-101). *Can. Invest.* 1987:5:545-552.

25. Thomas, P., Gangopadhyay, A., Steele, G., Andrews, C., Nakazato, H., Oikawa, S., and Jessup, J.M. The effect of transfection of the CEA gene in the metastatic behavior of the human colorectal cancer cell line MIP-101. *Can. Lett.* 1995:92:59-66.

26. Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevack, E.M., Strober, W., and Coico, R., eds. Current Protocols in Immunology. John Wiley and Sons, 1998.

27. Sperling, A.I., Auger, J.A., Ehst, B.D., Rulifson, I.C., Thompson, C.B., and Bluestone, J.A. Cd28/B7 interactions deliver a unique signal to naive T cells that regulates cell survival but not early proliferation. J. Immunol. 1996:157:3909-3917.

28. Fisher, B., Packard, B.S., Read, E.J., Carrasquillo, J.A., Carter, C.S., Topalian, S.L., Yang, J.C., Yolles, P., Larson, S.M., and Rosenberg, S.A. 1989. Tumor localization of adoptively transferred indium-111 labeled tumor infiltrating lymphocytes in patients with metastatic melanoma. *J. of Clin. Oncol.* 7:250-261.

29. Itoh, K., Sawamura, Y., Hosokawa, M., and Kobayashi, H. 19988. Scintigraphy with indium-111 labeled lymphokine-activated killer cells of malignant brain tumor. *Radiation Med.* 6:276-281.

30. Schafer, E., Dummer, R., Eilles, C., Borner, W., Martin, R., Rendl, J., and Burg, G. 1991. Imaging pattern of radiolabeled lymphokine activated killer cells in patients with metastatic malignant melanoma. *Eur. J. Nucl. Med.* 18:106-110.

31. Schwartzentruber, D.J., Hom, S.S., Dadmarz, R., White, D.E., Yannelli, J.R., Steinberg, S.M., Rosenberg, S.A., and Topalian, S.L. 1994. In vitro predictors of therapeutic response in melanoma patients receiving tumor-infiltrating lymphocytes and interleukin-2. *J. Clin. Oncol.* 12:1475-1483.

32. Hodge, J.W., Sabzevari, H., Yafal, A.G., Gritz, L., Lorenz, M.G.O. and Schlom, J. A triad of costimulatory molecules synergize to amplify T cell activation. Can. Res. 199:59:58005807.

33. Sprent, J. Stimulating naive T cells. J. Immunol. 1999:163:4629-4636.

34. Gong, M.C., Latouche, J-B., Krause, A., Heston, W.D.W., Bander, N.H., and Sadelain, M. Cancer patietn T cells genetically targeted to prostate-specific membrane antigen specificially lyse prostate cancer cells and release cytokines in response to prostate-specific membrane antigen. Neoplasia, 1999:1:123-127.

35. Fraser, J.D., Irving, B.A., Crabtree, G.R., and Weiss, A. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. Science 1991:251:313.

36. Jenkins, M.K., Taylor, P.S., Norton, S.D., and Urdahl, K.B. CD28 delivers a costimulatory signal involved in antigen specific IL2 production by human T cells. J. Immunol. 1991:147:2461.

37. Lindsten, T., June, C.H., Ledbetter, J.A., Stella, G., and Thompson, C.B. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. Science. 1989:244:339.

38. Mcheyzer-Williams, M.G., and Davis, M.M. Antigen-specific development of primary and memory T cells in vivo. Science. 1995:268:106-111.

39. Bachmann, M.F., Speiser, D.E., and Ohashi, P.S., Functional maturation of and antiviral cytotoxic T cell response. J. Virol. 1997:71:5764-5768.

40. Busch, D.H., Pilip, I., and Pamer, E.G. Evolution of a complex TCR repertoire during primary and recall bacterial infection. J. Exp. Med. 1998:188:61-70.

41. Busch, D.H., and Pamer, E.G. T cell affinity maturation by selective expansion during infection. J. Exp. Med. 1999:189:701-709.

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FIGURE LEGENDS

Fig. 1- Normal human T lymphocytes transduced with the anti-CEA IgTCR vector.

OKT3-activated human T cells were infected with the anti-CEA IgTCR vector (Methods), stained with either PE-labeled WI2 anti-IgTCR antibody (solid lines) or with UPC negative control antibody (dotted lines) and then analyzed by flow cytometry.

Fig. 2- Signal 1 stimulation causes AICD with plate-, or tumor-bound antigens.

T cells from Fig. 1 were incubated in either tissue culture plates coated with anti-IgTCR antibody, in co-cultures with MIP-CEA tumor cells, or in regular growth media with no antigenic stimulation as indicated in legend. Viable T cells were counted as described in the Methods section, and plotted as a function of time.

Fig. 3- Addition of signal 2 to signal 1 induces T cell proliferation.

T cells from Fig. 1 were incubated in IL2-containing growth media in tissue culture plates coated with either anti-IgTCR antibody alone, with anti-CD28 antibody alone, with anti-IgTCR and anti-CD28 antibodies together, or in IL2-containing media with no antigenic stimulation as indicated in the legend.

Viable T cells were counted (Methods), normalized to the number of cells present in the untreated controls, and plotted as percent of controls over time.

Fig. 4- Introduction of the human B7-1 gene into colon carcinoma cell lines. MIP-101 and MIP-CEA tumor cells were transduced with a retroviral vector containing the human B7-1 gene. The parental and transduced tumor cell lines were then stained with PE-labeled hMN14 (CEA expression, y-axis), and FITC-labeled anti-human B7-1 antibody (B7 expression, x-axis), or with the appropriate negative control antibody (HAT-PE and UPC-FITC). FACS analysis of cells stained with control antibodies (not shown) were used to set the position of the quadrants in each panel. Panel A: parental MIP-101 cells. Panel B: B7-1 transduced MIP-101 cells. Panel C: Parental MIP-CEA cells. Panel D: B7-1 transduced MIP-CEA cells.

Fig 5.- T cell proliferation in response to tumor cells that display various antigens.

T cells from Fig. 1 were coincubated with each of the tumor cell lines from Fig. 4 in IL2-containing growth media. Co-cultures were sampled at specific times and counted for both viable T cells and viable tumor cells. Panel A: Counts of viable T cells from co-cultures with MIP-CEA, or MIP-CEA-B7. Cultures terminated before day 27 were due to tumor cell overgrowth. Panels B and C: Counts of viable tumor cells from co-cultures with MIP-CEA (Panel B), or

MIP-CEA-B7 (Panel C). T cell numbers form panel A are separately reproduced in B and C for reference purposes.

Fig. 6- Proliferation of T cells stimulated with different antigens and at different signal strengths.

T cells from Fig. 1 were cultured in IL2-containing growth media in tissue culture plates coated with anti-IgTCR antibody, or with anti-IgTCR and anti-CD28 antibodies together as indicated in the legend. Cultures were assayed for cell proliferation after 10 days of stimulation. Wells were coated with anti-IgTCR antibody at the concentrations shown on the x-axis admixed with either a constant amount of anti-CD28 antibody (10 μg/ml), or with no anti-CD28 antibody. Zero values for anti-IgTCR antibody concentration on the x-axis represent either no antibody at all (anti-IgTCR only line), or with 10 μg/ml of anti-CD28 antibody alone (anti-IgTCR+anti-CD28 antibody line).

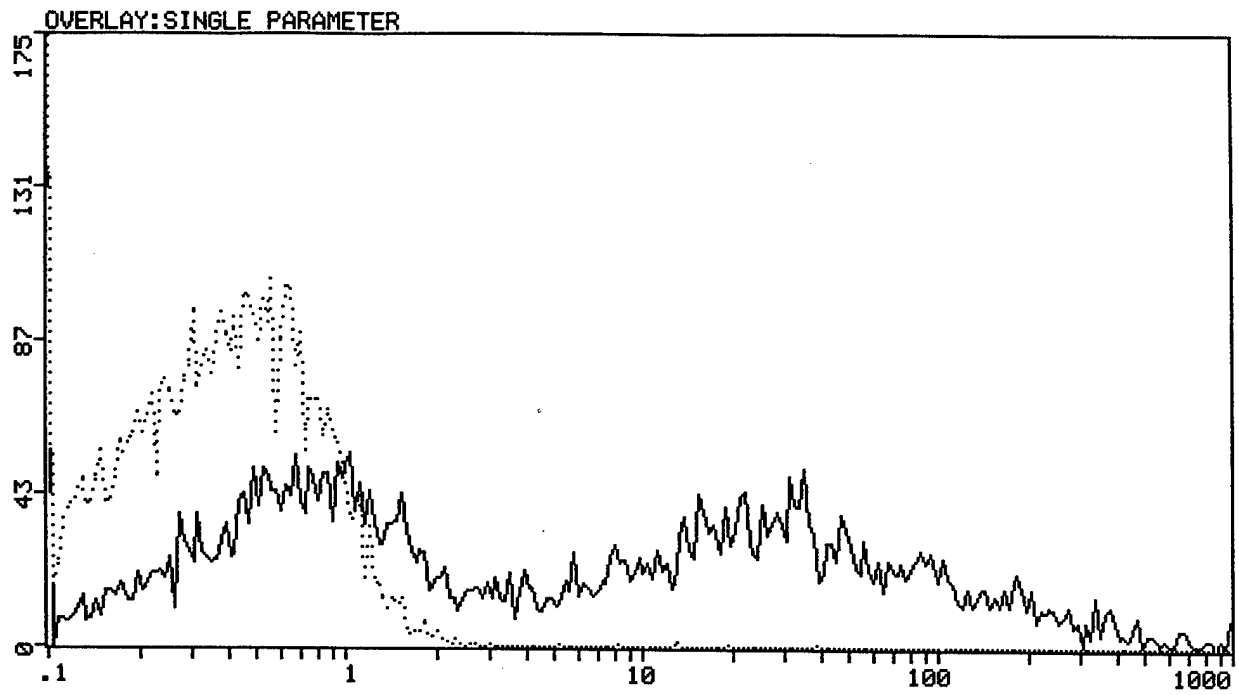
Fig. 7- T cell morphologic activation is controlled by signal 1.

T cells from Fig. 6 were analyzed by FACS for forward-scatter/log side-scatter. Panel A. Control cells not exposed to antibody. Panel B. Cells stimulated with IgTCR signal 1 only. Panel C. Cells stimulated with signal 1 and 2, but not proliferating (samples treated with 0.00001-0.01 ug/mL). Panel D. Cells stimulated with signal 1 and 2 that were proliferating (samples treated with 0.05-5 ug/mL). Dead and dying cells are indicated with an arrows, and viable

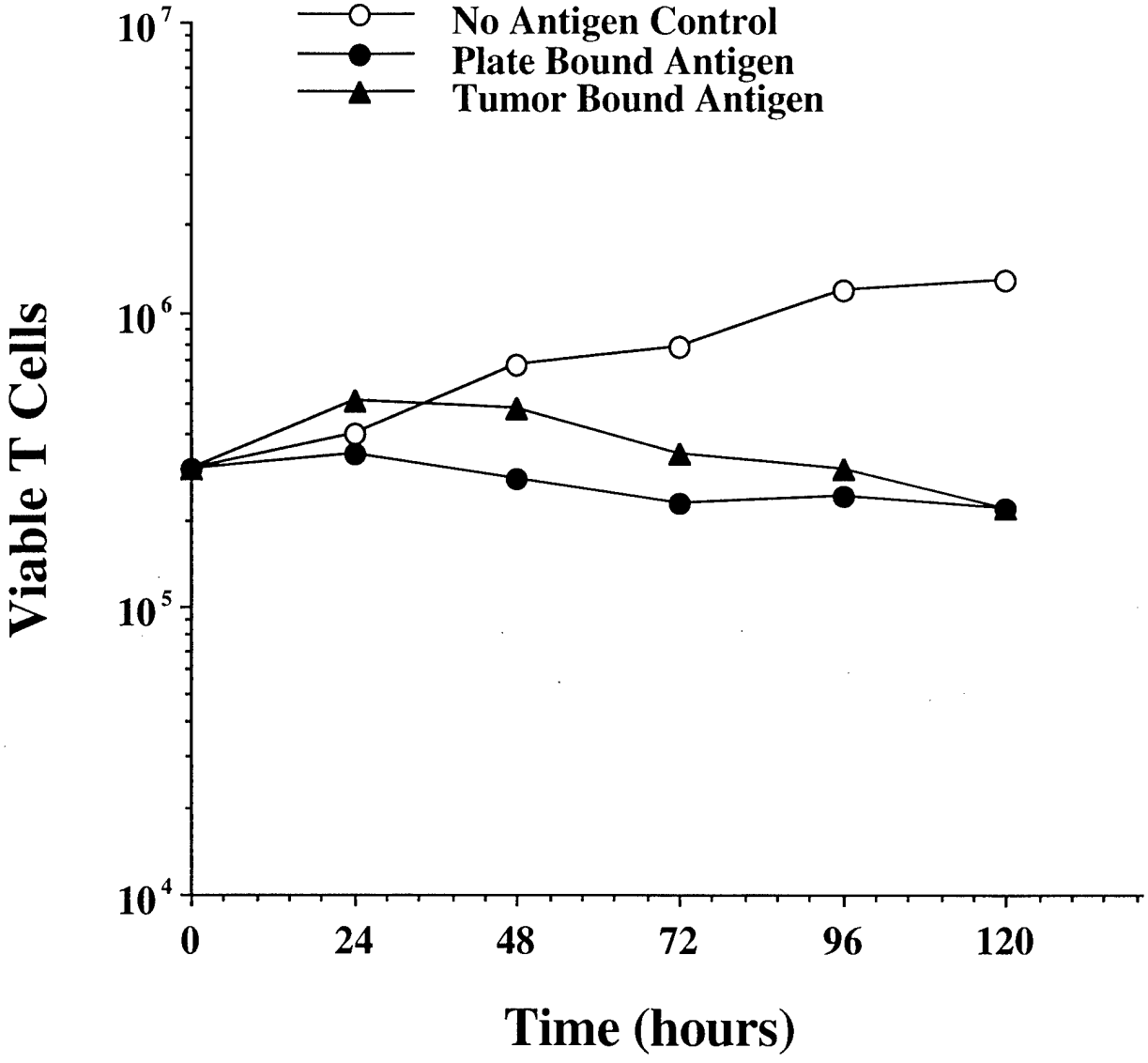
cells are circled.

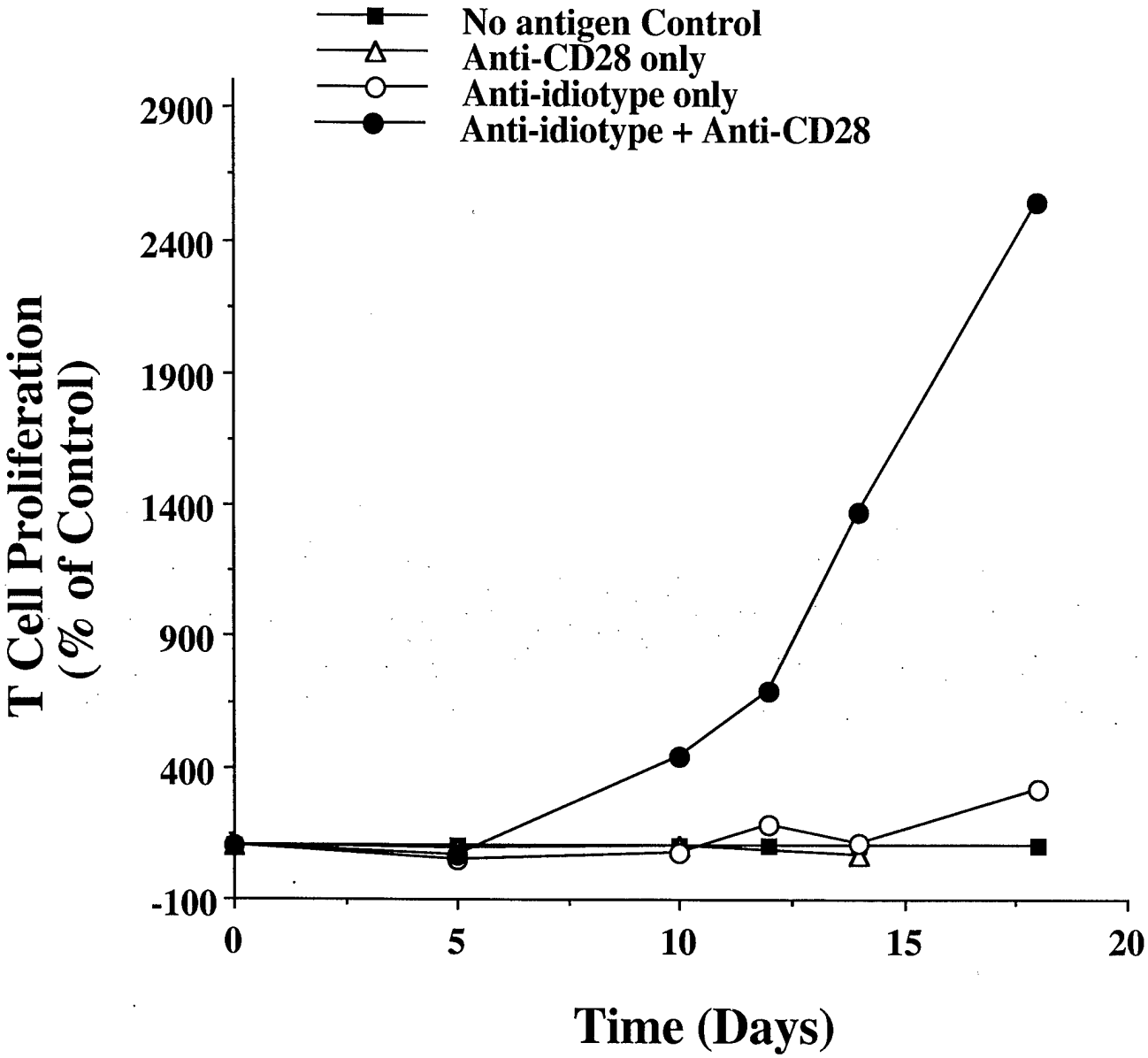
Fig. 8- Complete signals lead to clonal expansion of reactive clones, while incomplete signals induce clonal deletion by AICD.

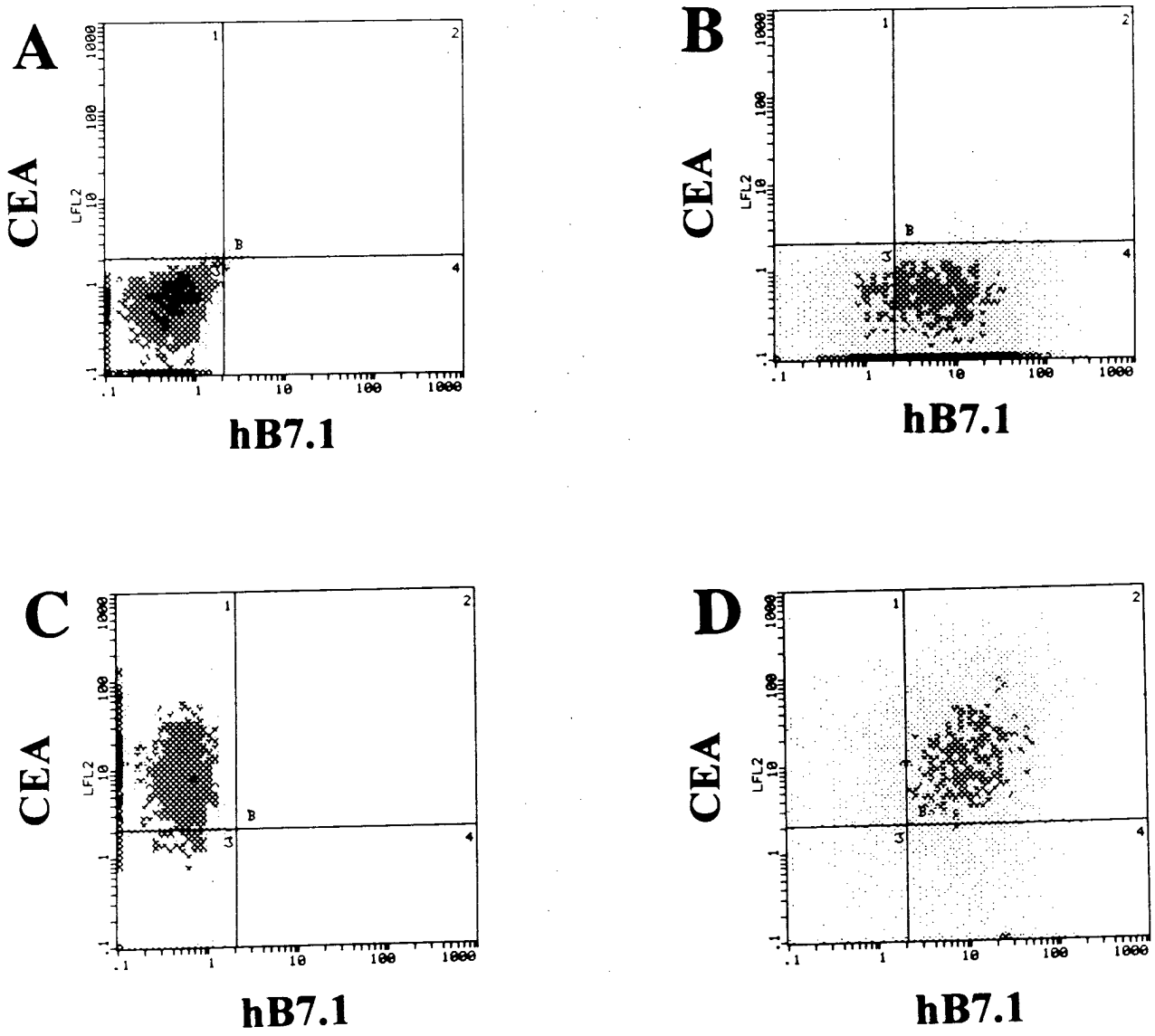
T cells were stimulated for 5 and 10 days with signal 1 only, or with signal 1 and 2 as labeled in the figure. Cells were then stained with PE-labeled anti-IgTCR antibody (solid lines) and assayed for IgTCR expression by FACS. Dotted lines are the negative antibody staining controls.

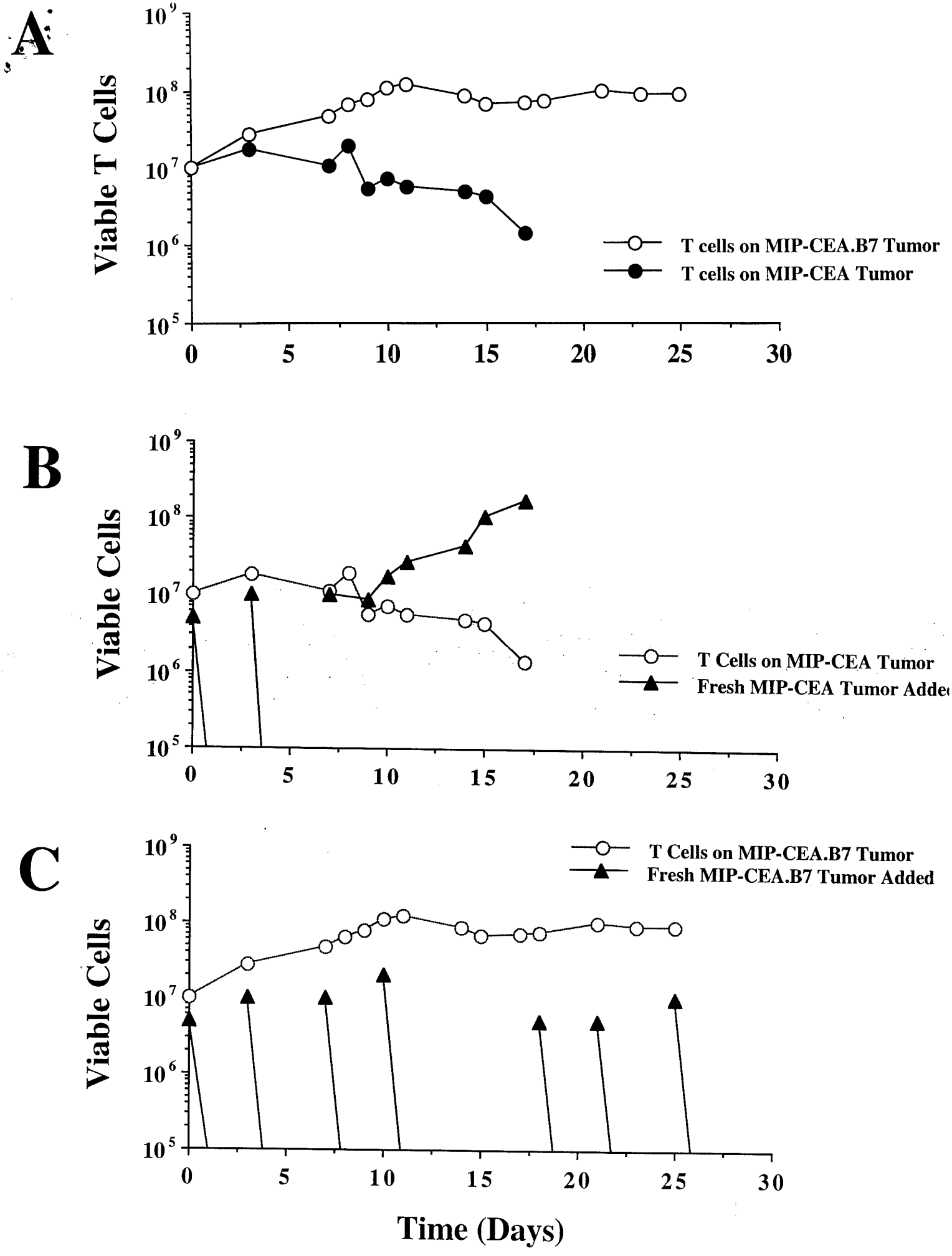


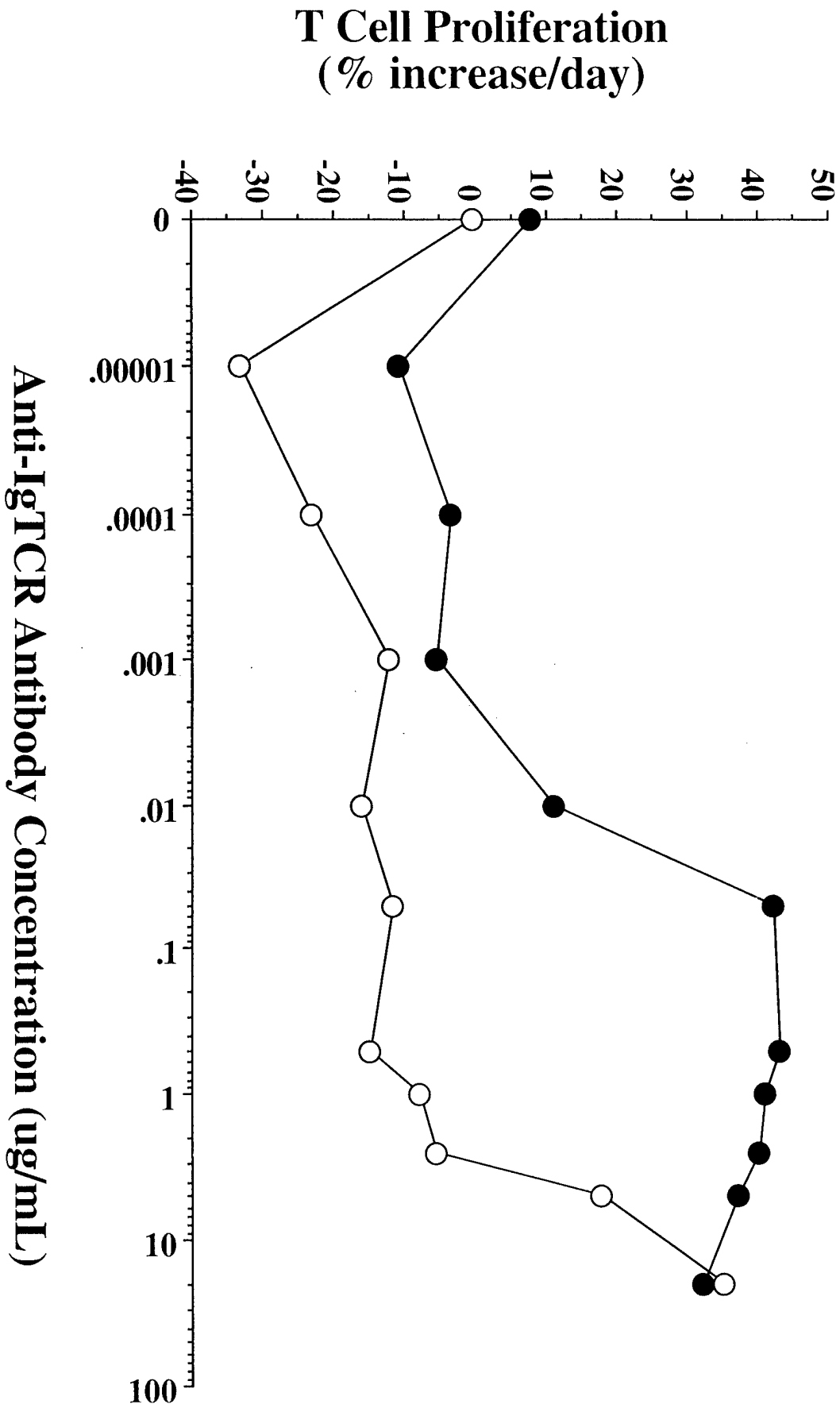
IgTCR

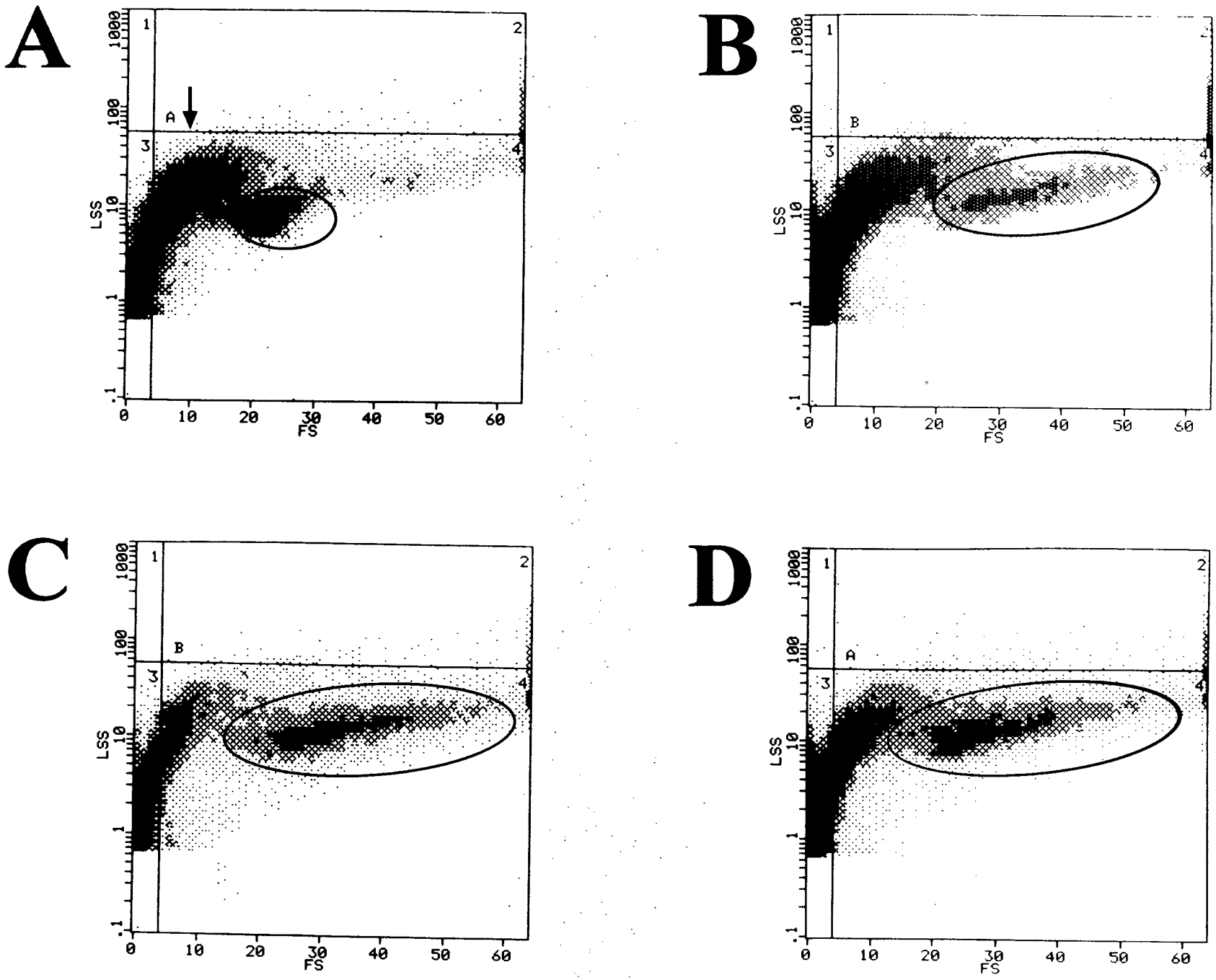

No Antigen Control
Plate Bound Antigen
Tumor Bound Antigen
Viable T Cells
10^7
10^6
10^5
10^4
0
24
48
72
96
120
Time (hours)

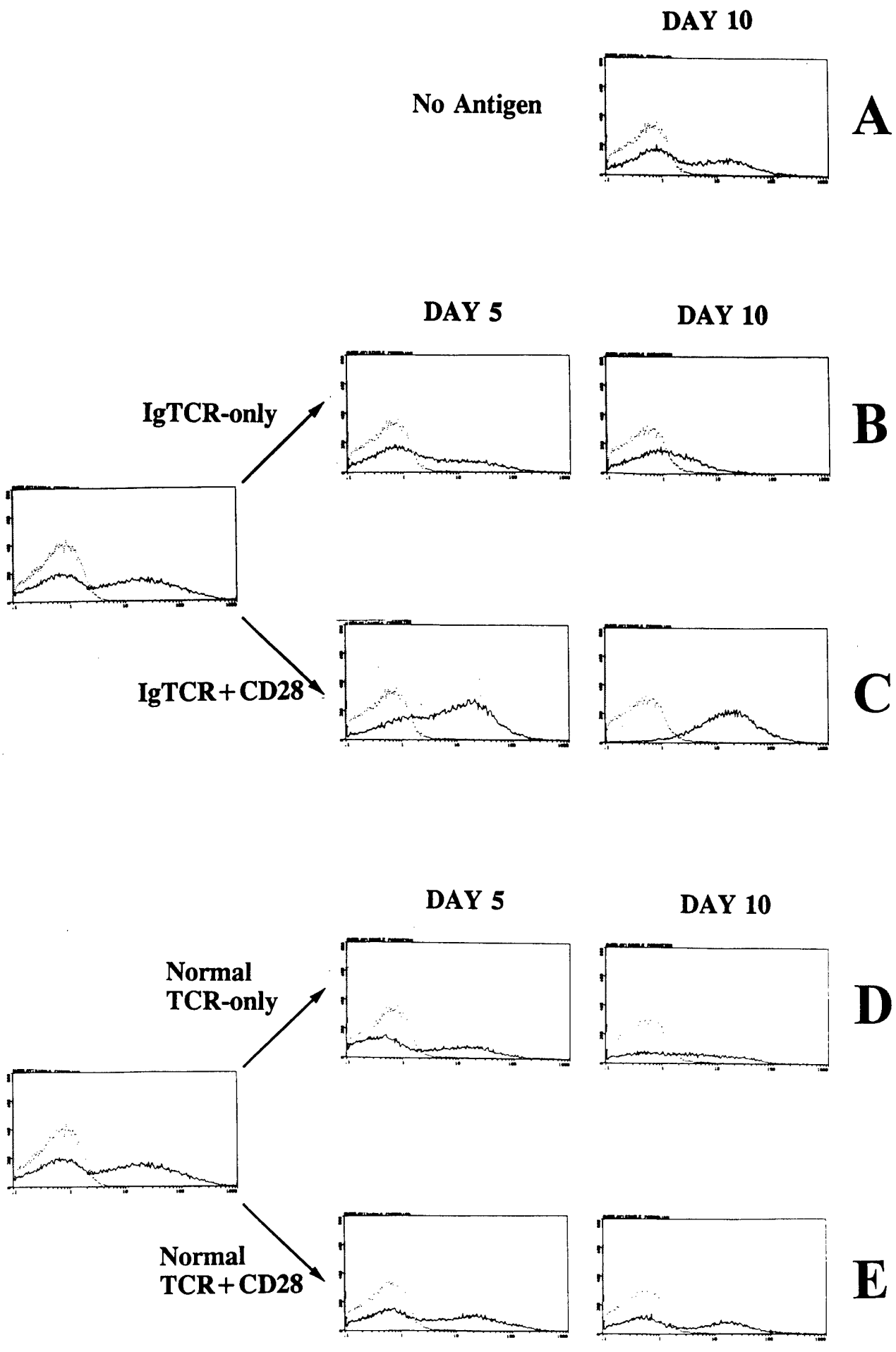

No antigen Control
Anti-CD28 only
Anti-idiotype only
Anti-idiotype + Anti-CD28
T Cell Proliferation
(% of Control)
2900
2400
1900
1400
900
400
-100
0
5
10
15
20
Time (Days)


A
CEA
LFL2
1
2
B
3
4
.1
1
10
100
1000
hB7.1
B
CEA
LFL2
1
2
B
3
4
.1
1
10
100
1000
hB7.1
C
CEA
LFL2
1
2
B
3
4
.1
1
10
100
1000
hB7.1
D
CEA
LFL2
1
2
3
4
.1
1
10
100
1000
hB7.1


A
Viable T Cells
10^9
10^8
10^7
10^6
10^5
0
5
10
15
20
25
30
T cells on MIP-CEA.B7 Tumor
T cells on MIP-CEA Tumor
B
Viable Cells
T Cells on MIP-CEA Tumor
Fresh MIP-CEA Tumor Adde
C
Viable Cells
T Cells on MIP-CEA.B7 Tumor
Fresh MIP-CEA.B7 Tumor Added
Time (Days)


T Cell Proliferation
(% increase/day)
-40
-30
-20
-10
0
10
20
30
40
50
0
.00001
.0001
.001
.01
.1
1
10
100
Anti-IgTCR Antibody Concentration (ug/mL)
Anti-IgTCR only
Anti-IgTCR+Anti-CD28


A
B
C
D
LSS
FS
1000
100
10
1
.1
0
10
20
30
40
50
60
1
2
3
4
A
B


DAY 10
No Antigen
A
DAY 5
DAY 10
IgTCR-only
B
IgTCR+CD28
C
DAY 5
DAY 10
Normal
TCR-only
D
Normal
TCR+CD28
E